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Master's Degree Thesis

Severe Acute Respiratory Syndrome Coronavirus 2: Development and Evaluation of Diagnostic Techniques with Analysis of COVID-19 Risk Factors.

Graduate School of Chosun University

Department of Medicine

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Advisor: Professor, Dong-Min Kim, MD, Ph.D

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Dedicated to my late father-in-law Muhammad Younas, my mother-in-law Rukhsana Younas, my father Muhammad Tariq and my mother Atiya Tariq.

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Nomenclatures

1,25(OH)$_2$ D	1,25-dihydroxyvitamin D
25(OH) D	25-hydroxyvitamin D
Ag-RDTs	Rapid antigen detetction rapid diagnostic tests
ANOVA	One-way analysis of variance
ARDS	Acute respiratory distress syndrome
AUC	Area under the ROC curve
AuNPs	Gold nanoparticles
BALF	Bronchoalveolar lavage fluid
cDNA	Complementary DNA
CI	Confidence intervals
CKD	Chronic kidney disease
CLD	Chronic liver disease
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CPE	Cytopathic effect
Ct	Cycle threshold
DMEM	Dulbecco's modified Eagle's medium
E	Envelop
ELISA	Enzyme linked immunosorbent assay
FIA	Fluorescence immunoassay
HRP	Horseradish peroxidase
IFN-I	Type I Interferon

IQR	Interquartile range
M	Membrane
N	Nucleocapsid protein
PBS-T	Phosphate-buffered saline with Tween 20
PSO	Post-symptom onset
PVDF	Polyvinylidene difluoride
RdRP	RNA-dependent RNA polymerase
rNP	Recombinant nucleocapsid protein
ROC	Receiver operating characteristic
rRT-PCR	Real-time reverse transcription-polymerase chain reaction
RT–LAMP	Reverse-transcription loop-mediated isothermal amplification
S	Spike
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SpO2	Oxygen saturation
TMB	3,3´,5,5′-tetramethylbenzidine substrate

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Abstract

Severe Acute Respiratory Syndrome Coronavirus 2: Development and Evaluation of Diagnostic Techniques with Analysis of COVID-19 Risk Factors

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Background

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has expanded over the world since the first case was identified in December 2019, posing significant public health risks. Rapid identification and effective isolation are crucial for curbing the spread of SARS-CoV-2. To meet this requirement, we developed a serological assay based on SARS-CoV-2 recombinant nucleocapsid protein (rNP) and compared it to three commercial ELISAs: STANDARDTM E COVID-19 Total Ab ELISA, EDI^{TM} Novel Coronavirus COVID-19 IgG, and IgM ELISA. We also aimed to evaluate the performance of rapid antigen detetction rapid diagnostic tests (Ag-RDTs). In addition, we analyzed the various risk factors affecting the severity of COVID-19. In particular, we analyzed vitamin D metabolites (25-hydroxyvitamin D [25(OH) D] and 1,25-dihydroxyvitamin D [1,25$(OH)_2$ D]) along with potential demographic risk factor influencing the outcome and severity of COVID-19.

Methods

Plants- and *Escherichia coli*–based rNP protein was expressed, purified and used for the

detection of serum total antibody (Ab). In addition, comparative analysis was performed with the commercially available diagnostic kits. The clinical performance of Ag rapid fluorescence immunoassay (FIA) and Ag Gold was evaluated and compared in parallel with genomic and subgenomic real-time reverse transcription-polymerase chain reaction (rRT-PCR) and cell culture-based assays. Patients were divided into four groups based on the severity of their infection: asymptomatic, mild to moderate, severe, and critical. Serum 25(OH) D and $1,25(OH)_2$ D concentrations, as well as serum ferritin, CRP, and D-dimer, were also measured. Patients' demographic characteristics, such as gender, age, BMI, and co-morbidities, were also compared.

Results

As a result of evaluating the accuracy of ELISA assays, the sensitivity and specificity were as follows: 92.91% / 94.30% (plant-rNP), 83.69% / 98.73% (SD Biosensor), 75.89% / 98.10% (*E. coli*-rNP), 76.47% / 100% (EDI-IgG), and 80.39% / 80% (EDI-IgM). Among all the assays, the plant-based rNP showed the highest sensitivity and area under the ROC curve (0.980) ($p < 0.05$). For both rNP-plant-based and SD Biosensor ELISAs, the seroconversion rate for total Ab progessively increased with disease progression, with a sensitivity of 100% after 10-12 days of post-symptom onset (PSO). After 2 weeks of PSO, the seroconversion rates were > 80% and 100% for EDI-IgM and IgG ELISA, respectively. Seroconversion occurred earlier with rNP plant-based ELISA (5 days PSO) compared with *E. coli*-based (7 days PSO) and SD Biosensor (8 days PSO) ELISA. In case of Ag-RDTs, for rRT-PCR-positive samples, the detection sensitivity of Ag rapid FIA and Ag Gold was 74.51% and 53.49%, respectively, with a specificity of 100%; however, for samples with low cycle threshold (Ct) values, Ag rapid FIA and Ag Gold exhibited a sensitivity of 82.61% ($Ct \leq 30$, 5.6 $\log_{10}$RNA copies/mL) and 80% ($Ct \leq 25$, 6.9 $\log_{10}$RNA copies/mL), respectively. Despite low analytical sensitivity, both Ag-RDTs detected 100% infection in cell culture-positive samples (n = 15) and were highly effective in distinguishing viable samples from those with subgenomic RNA (66.66%). According to thr nutritional staus of COVID-19 patients, vitamin deficiency [25 (OH) D < 20ng/mL] was present in 59% of all patients. However, based on

the severity of illness the serum concentration of 25(OH) D and $1,25(OH)_2$ D did not differ significantly between the four groups ($p = 0.478$ and 0.358, respectively). Furthermore, survival analysis revealed no significant differences in mean 30-day survival when different levels of both vitamin D metabolites were analyzed ($p = 0.181$ and 0.164). Increased co-morbidities were observed in severe and critical groups ($p = 0.002$). The mean age, CRP, and serum ferritin levels differed significantly between four groups ($p < 0.01$).

Conlcusions

We observed that in house ELISA with plant-derived rNP can reliably detect SARS-CoV-2 total Abs. The assay can be utilized for COVID-19 serological investigations and further diagnosis. Our findings demonstrate that Ag-RDTs successfully identify viable SARS-CoV-2, suggesting that they might be a useful technique for rapidly diagnosing transmissible infected individuals. Although vitamin D deficiency was observed in 59% of all COVID-19 patients, we found no statistically significant relationship between vitamin D and COVID-19 severity and fatality.

초록

중증급성호흡기증후군 코로나바이러스 2: COVID-19 위험요인 분석과 진단법 개발 및 평가

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배경

중증급성호흡기증후군 코로나바이러스 2(SARS-CoV-2) 대유행은 2019년 12월 첫 번째 사례가 확인된 이후 전 세계로 확대되어 심각한 공중 보건 위험을 제기하고 있다. 신속한 진단과 효과적인 격리는 중증 급성 호흡기 증후군 코로나바이러스 2(SARS-CoV-2)의 확산을 억제하는 데 중요하다. 이 요구 사항을 충족하기 위해 연구자는 SARS-CoV-2 재조합 뉴클레오캡시드 단백질을 기반으로 한 혈청학적 분석법을 개발하고 이를 세 가지 상용 ELISA: STANDARDTM E COVID-19 Total Ab ELISA(SD BIOSENSOR), EDITM 신종 코로나바이러스 COVID-19 IgG 및 IgM ELISA 와 비교하였다. 또한 신속 항원 검출 신속 진단 검사(Ag-RDT)의 성능을 평가하는 것을 목표로 삼았다. 또한, 코로나 19의 중증도에 영향을 미치는 다양한 위험인자를 분석하였고 특히 비타민 D 대사 산물

(25-하이드록시비타민 D[25(OH) D] 및 1,25-디하이드록시비타민 D[1,25$(OH)_2$ D])의 상태와 함께 COVID-19의 중증도에 미치는 영향을 분석하였다.

방법

식물 및 대장균 기반 재조합 뉴클레오캡시드 단백질을 발현, 정제하여 혈청 총 항체(total Ab)의 검출에 사용하였다. 또한 코로나 19 진단에서의 임상적 유용성을 평가하기 위해 상업적으로 판매되는 진단 키트와 비교분석하였다. 전염 가능한 배양양성 환자의 신속한 진단에 있어서 신속항원검사와 서브게놈 실시간 역전사 중합효소 연쇄반응의 임상적 유용성을 평가하기 위해 Ag Rapid Fluorescent Immunoassay(FIA) 및 Ag Gold를 이용한 신속항원검사와 서브게놈 실시간 역전사 중합효소 연쇄 반응(rRT-PCR) 및 세포 배양 기반 분석을 병행하여 평가 및 비교하였다. 환자는 감염의 중증도에 따라 무증상, 경증에서 중등도, 중증 및 위독의 4개 그룹으로 분류하였다. 중증도와 관련된 인자 평가를 위해 혈청 25(OH) D 및 1,25$(OH)_2$ D 농도뿐만 아니라 혈청 페리틴, CRP 및 D-이량체를 측정하였고, 성별, 연령, 체질량지수(BMI) 및 동반 질환과 같은 환자의 인구 통계학적 특성도 비교하였다.

결과

연구자가 개발한 효소면역분석법 (ELISA)의 정확도 평가결과 민감도와 특이도는 92.91%/94.30%(plant-rNP), 83.69% / 98.73%(SD Biosensor), 75.89% / 98.10%(*E. coli*-rNP), 76.47% / 100%(EDI- IgG) 및 80.39% / 80%(EDI-IgM)를 보였다. 식물에서 합성한 rNP를 이용한 효소면역분석법이 가장 높은 민감도와 ROC 곡선 아래 면적(0.980)($p <$

0.05)을 보였다. rNP 식물 기반 및 SD 바이오센서 ELISA 둘 다 총 Ab에 대한 혈청전환율은 질병 진행에 따라 점진적으로 증가했으며, 증상 발병 후 10-12일 후 민감도는 100%였다. PSO 2주 후, 혈청전환율은 EDI-IgM 및 IgG ELISA에 대해 각각 > 80% 및 100%였다. 대장균 기반(7일 PSO) 및 SD Biosensor(8일 PSO) ELISA에 비해 rNP 식물 기반 ELISA(5일 PSO)에서 혈청전환이 더 일찍 확인되었다. 신속항원검사의 경우 rRT-PCR 양성 샘플의 경우 Ag Rapid FIA 및 Ag Gold의 검출 민감도는 각각 74.51% 및 53.49%였으며, 특이도는 100%였다. 그러나 낮은 주기 임계값(Ct)을 가진 샘플의 경우 Ag Rapid FIA 및 Ag Gold는 82.61%(Ct ≤ 30, 5.6 log_{10}RNA 사본/mL) 및 80%(Ct ≤ 25, 6.9 log_{10}RNA 사본/mL)의 감도를 나타냈다. 각각. 낮은 분석 감도에도 불구하고 Ag-RDT는 모두 세포 배양 양성 샘플(n = 15)에서는 100% 감염을 감지했으며, 바이러스 배양된 샘플을 검출하는데 서브게놈 실시간 역전사 중합효소 연쇄 반응의 민감도는 66.66%를 보여 신속항원검사가 더 민감하게 검출 가능함이 확인되었다. 동반질환이 있을 경우 중증으로 진행이 관찰되었으며(p = 0.002), 평균 연령, CRP 및 혈청 페리틴 수치는 4개 그룹 간에 유의한 차이가 있었다(p < 0.01). 코로나19 환자의 내원당시의 비타민 D의 농도 분석에 따르면 전체 환자의 59%에서 비타민 결핍[25(OH) D < 20 ng/mL]이 확인되었다. 그러나 질병의 중증도에 따른 25(OH) D 및 $1,25(OH)_2$ D의 혈청 농도는 4개 그룹 간에 유의한 차이가 없었다(각각 p = 0.478 및 p = 0.358). 또한, 생존 분석에서는 두 비타민 D 대사 산물의 농도에 따른평균 30일 생존률 분석에 있어서 농도에 따른 유의한 차이가 없음을 보여주었다(p = 0.181 and 0.164).

결론

식물에서 발현된 rNP를 이용한 효소면역분석법이 SARS-CoV-2 총 Ab를 효과적으로 검출할 수 있음을 확인하였다. 이 분석은 COVID-19 혈청 조사 및 추가 진단에 활용될 수 있다. Ag-RDT가 전염가능한 SARS-CoV-2 감염환자를 신속하게 진단하여 감염된 사람들을 신속하게 격리조치하는데 유용한 기술이 될 수 있음을 확인하였다. 모든 코로 COVID-19 환자의 59%에서 비타민D 결핍이 관찰되었지만 비타민 D 와 COVID-19 중증도 및 사망률 사이에 통계적으로 유의미한 연관성이 없음을 확인하였다.

I. Introduction

A. Background

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), commonly known as the coronavirus, is the virus that causes coronavirus disease 2019 (COVID-19), the respiratory disease that is the cause of the continuing COVID-19 pandemic. The virus was formerly known as 2019 novel coronavirus (2019-nCoV), and it has also been referred to as human coronavirus 2019 (HCoV-19 or hCoV-19) [2]. The outbreak was first discovered in the city of Wuhan, Hubei, China, and the World Health Organization designated it a Public Health Emergency of International Concern on January 30, 2020, then a pandemic on March 11, 2020 [3].

There are now seven human coronaviruses, hCoV-229E and hCoV-NL63 are two alpha-coronaviruses, whereas hCoV-HKU1, hCoV-OC43, SARS-CoV, MERS-CoV, and SARS-CoV-2 are five beta-coronaviruses. The hCoV-229E, hCoV-NL63, hCoV-OC43, and hCoV-HKU1 infected patients simply have a typical cold [4]. SARS-CoV, MERS-CoV, and SARS-CoV-2, on the other hand, induce severe acute respiratory syndrome. SARS-CoV-2 is a positive-sense single-stranded RNA virus, belongs to the beta-coronavirus genus and Coronaviridae family, with a genome of nearly 30,000 nucleotide [5]. The SARS-CoV-2 is mainly composed of four structural proteins namely spike (S), nucleoprotein (N), envelope (E), and membrane (M) proteins [6]. These proteins play a vital role in viral genome synthesis, replication, virion-receptor attachment, virion and viroporin development, and eventually enhance viral entrance and proliferation, consequently spreading the infection.

B. Clinical presentation and risk factors

Majority of patients with COVID-19 presents with fever, shortness of breath, myalgia and fatigue, while headache and diarrhea are less common. A subset of patients presents with

radiographic evidence of pneumonia including numerous mottling and ground glass opacities [7]. However, critically ill individuals develops more catastrophic consequences, such as acute respiratory distress syndrome (ARDS) and cytokine strom, which may contribute to COVID-19 associated mortality [8, 9]. While the lungs are the major viral target, infection can also compromise the cardiovascular, brain, kidney, liver, and immunological systems [10]. The case fatality rate of COVID-19 is predicted to be between 3.4% and 11% [11]. The potential established host risk factors for severe outcome include advanced age, male gender, obesity, comorbidities or chronic illness [12, 13]. Due to substantial morbidity and mortality associated with COVID-19, social distance, facemasks, contact isolation, and hand hygiene are critical to reducing SARS-CoV-2 transmission. In addition, prompt clinical evaluation and identification of risk factors for poor outcomes are critical for treatment [14].

C. Diagnostic techniques and challanges

In current SARS-CoV-2 pandemic, reliable, early, and accurate identification is critical for providing prompt medical care to infected persons as well as assisting government agencies in preventing its spread to other individuals and saving people's lives. False negative test results may result in the spread of the disease in the population; similarly, false positive test results may result in needless therapy and mental torment for the patients. As a result, there is an urgent need for an accurate, fast, easily accessible, and dependable diagnostic test for SARS-CoV-2 infection. Various immunological and nucleic acid amplification diagnostic assays have been developed and made widely available thus far. Several integrated point-of-care molecular devices are presently being developed, and some are now available to enable accurate and quick diagnosis of SARS-CoV-2 infections.

D. Objective and scope of the study

The overall objective of this research is to establish a diagnostic technique based on an in-house enzynme linked linked immunosorbent assay (ELISA). The assay's diagnostic capability has been demonstrated by a comparison of recombinant nucleoproteins (rNP) expressed from plants and *E.coli* for the detection of total antibodies (total Ab) against SARS-CoV-2. We next compared the performance of our in-house developed assay to that of commercially available diagnostic kits (SD Biosensor STANDARD™ E COVID-19 Total Ab ELISA SD Biosensor STANDARD™ E COVID-19 Total Ab ELISA) in terms of sensitivity and specificity. We also evaluated a point-of-care test, the antigen detection rapid diagnostic test (Ag-RDT), and compared its clinical performance with real-time reverse transcription-polymerase chain reaction (rRT-PCR), including genomic and subgenomic RNA (sgRNA) PCR, and cell-culture assays, to provide comprehensive correlation with each diagnostic platform. We also evaluated COVID-19 risk variables based on infection severity. Demographic characteristics were compared with other laboratory parameters, as well as the mortality rate in patients in relation to status of vitamin D metabolites (25-hydroxyvitamin D [25(OH) D] and 1,25-dihydroxyvitamin D [$1,25(OH)_2$ D]). This study will aid in understanding the serological response in COVID-19 patients, as well as the probable relationship between disease severity and fatal outcomes.

II. Overview of SARS-CoV-2 detection techniques

The quick and precise identification of SARS-CoV-2, facilitated by real-time reverse transcription–polymerase chain reaction (RT–PCR), is the first step towards controlling COVID-19 [15]. SARS-CoV-2 nucleic acids are detected in the upper respiratory tract specimens by RT–PCR. Testing is used to keep infectious diseases from spreading between people and communities including asymptomatic infected people, whose viral shedding can accidentally transfer the illness to the elderly and others with disease comorbidities [16]. The need to improve test sensitivity and specificity remains important. Serological testing adds to viral detection by identifying previous infection, which might be used for therapeutic purposes. Antibodies are identified utilizing a qualitative detection of IgG or IgM antibodies in an enzyme-linked immunosorbent technique [17]. These assays measure an immune response to the viral S protein and N protein and can be used to detect protection against future viral exposure and/or contact tracing [18]. This holds true for epidemiological assessments as well as large global treatment requirements. The development of diagnostic assays to enhance immunoassay sensitivity and specificity will be a focus of future research [19]. As reinfections occur, such testing will eventually demonstrate viral protection. The next step in COVID-19 control is to induce immunity against SARS-CoV-2 [19, 20].

The identification of genomic RNA has aided in the early diagnosis of individuals infected with SARS-CoV-2. However, because genomic RNA detection cannot distinguish between viable and non-viable viruses, patients with SARS-CoV-2 infection must be isolated for longer periods of time, and contacts with those with SARS-CoV-2 infection must be quarantined despite the fact that they have a lower risk of transmitting the virus. The best indicator of the existence of replicating SARS-CoV-2 virus is culture-based isolation, although it is complex, labor-intensive, and time-consuming. The assay is further difficult by the requirement for Bio-safety Level 3 facilities. Coronaviruses, on the other hand, have a unique method for discontinuous transcription that involves the production of subgenomic RNA [21]. In coronavirus subgenomic RNA , a nested set of negative-sense RNAs from the 3´ end of the viral genome is connected to a common leader

sequence of around 70 nucleotides obtained from the 5´ end of the genomic RNA [22]. As a result, it is hypothesized that subgenomic RNA detection may better reflect replication-competent virus than standard genomic RNA detection [23].

A. Body fluids and tisuue distribution of SARS-CoV-2

The viral load of SARS-CoV-2 and viral particles in the respiratory tract follow virus dynamics in bodily fluids and tissue. All of these things have an impact on the host's immunological responses at the same time [24]. The quantity of virus in each sample varies, with respiratory, stool, and serum samples revealing large differences in viral levels [9]. The cell-specific expression of angiotensin converting enzyme-2 (ACE2) receptors is connected to the spread of infection from the respiratory tract to other tissues and organs [25]. The viral load in respiratory samples is greatest in the early stages of the illness, peaking in the second week, and then decline. As evidenced by throat and anal swab sample tests, virus persistence is constant [26] in individuals with comorbidities. Viral RT–PCR testing in throat swabs from disease-free people reveal positive findings for up to 50 days, and viral RNA has been detected in faecal and anal swabs weeks after respiratory samples have been confirmed negative. Overall, viral dynamics in hospitalized patients should be taken into account when making COVID-19 preventive and treatment recommendations [27].

B. Detection of SARS-CoV-2 viral shedding

The viral shedding in throat swabs and sputum peaks five to six days after symptom onset and ranges from 10^4 to 10^7 copies/mL. This is because viral levels in the respiratory tract are higher [28]. The rate of viral RNA detection in infected people's nasal swabs has approached 100%. Blood, saliva, and tears had positive rates of 88, 78, and 16 percent, respectively. Large-scale population field-testing using the chemiluminescence immunoassay, enzyme-linked immunosorbent, and

lateral-flow immunochromatographic assays is facilitated by self-collection of naso- or oropharyngeal swabs [29]. To offer a fast platform for point-of-contact serological detection, the lateral-flow immunochromatographic assay employs gold nanoparticles (AuNPs) and a colorimetric label. Here, the antigen of SARS-CoV-2 is coupled with nanoparticles [30]. When blood or saliva specimens are loaded SARS-CoV-2 IgG and IgM can bind to the SARS-CoV-2 antigen and antibody, which is identified colorimetrically (오류! 참조 원본을 찾을 수 없습니다.). The test takes 20 minutes to complete and is accurate to 90%. To present, viral shedding lasts at least 7 days following the beginning of symptoms, with viral infectivity occurring within 24 hours [31]. SARS-CoV-2 detection drops to undetectable levels when serum neutralizing antibodies are present [31]. Even in instances with concurrently high viral levels, the live virus could not be propagated in cell culture 8 days after symptom onset. These findings support the use of quantitative viral RNA load and serological tests, when choosing whether to discontinue infection control measures [1].

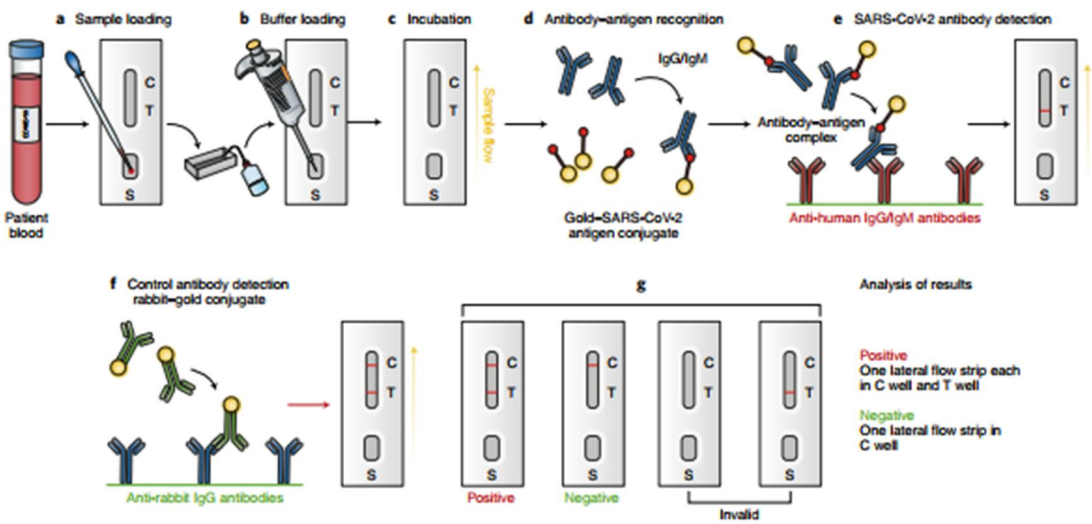


Figure 1: Serological testing for SARS-CoV-2 [1]

Serological testing for SARS-CoV-2. SARS-CoV-2-specific recombinant antigens mounted on nitrocellulose membranes are often utilized in immune-based testing. Mouse anti-human IgM and IgG antibodies are immobilized on conjugate pads with coloured latex beads. Within the test, the

test sample comes into contact with the membrane. The colored antibodies combine with human antiviral antibodies to create latex conjugate complexes. The SARS-CoV-2-specific recombinant antigen captures this complex fixed on the membrane. A coloured band appears if SARS-CoV-2-specific IgG/IgM is present in the sample, suggesting a positive test result. A red control line is formed on the membrane when the complex is caught by goat anti-mouse antibody. In the test window, there is a built-in control line. A negative result is shown by the lack of a colored band. **a–e**, The procedure starts with the addition of patient serum to the sample flow well (S) **(a),** Dropwise saline buffer is added **(b)**, and the sample is incubated **(c)** until antibody–antigen identification **(d)** and SARS-CoV-2 antibody identification **(e). f,** In the control (C) well, the rabbit antibody–gold is visible. **g,** The presence of COVID-19 antibody is indicated by a positive test (T) band, and findings without a positive C band are invalid. This test, in particular, represents a post-immune response and may yield negative findings in those who have just been infected. It may also identify virus in those who have been infected but are asymptomatic.

C. Real-time reverse transcription-polymerase chain reaction (rRT-PCR)

rRT-PCR successfully amplifies extremely little quantities of viral genetic material in a combination of other nucleic acid sequences and is presently the gold standard technology for detecting SARS-COV-2 in upper respiratory tract samples [15]. The reverse transcriptase first transforms the RNA viral genome into DNA using a short DNA sequence primer, followed by the production of complementary DNA (cDNA). The amplification of DNA is monitored in real time using a fluorescent dye or a fluorescence-labeled sequence-specific DNA probe. After several amplification rounds, a fluorescent or electrical signal shows the viral cDNA [32] (**오류! 참조 원본을 찾을 수 없습니다.**). RT-PCR technique has been utilized to diagnose SARS-CoV-2 using genomic areas such as ORF1b and ORF8, as well as the nucleocapsid (N), RNA-dependent RNA polymerase (RdRP), spike (S) protein, and envelope (E) genes [33].

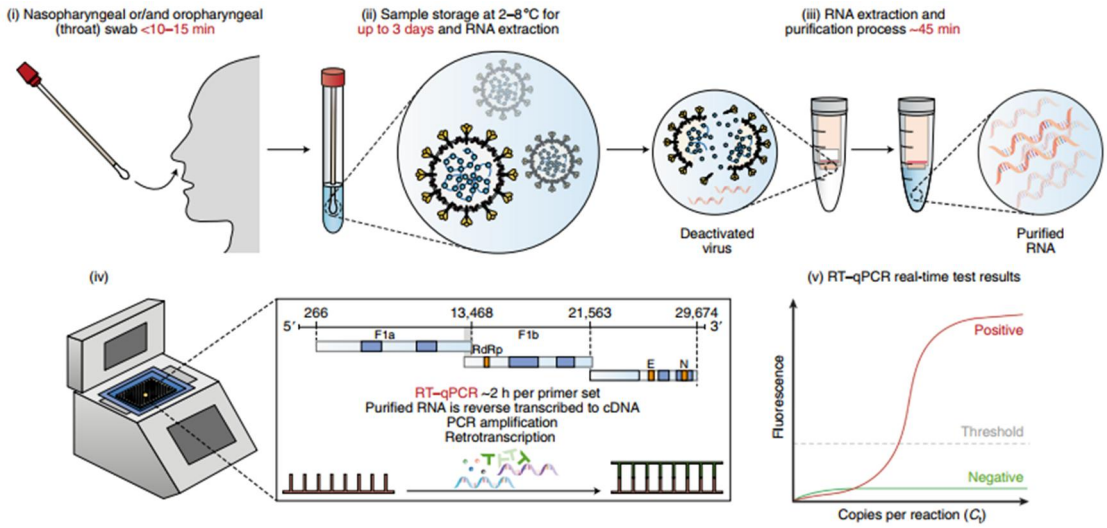


Figure 2: Real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay [1]

(i) Patient samples are collected using a nasopharyngeal swab. **(ii), (iii)** RNA is isolated from fluids containing SARS-CoV-2-infected cells and free virus particles. **(iv)** The viral RNA is subsequently reverse transcribed into cDNA and amplified for viral nucleic acid identification. The subgenomic viral segments amplified with a fluorogenic probe by qPCR are the conserved portions of the RdRp and E genes. **(v)** Positive instances exceed the detection threshold.

D. Serological detection of SARS-CoV-2

Antibodies against SARS-CoV-2 are produced as a main immunological response to infection. By day 7, up to 50% of infected people have neutralizing antibodies, and by day 14, all infected individuals have neutralizing antibodies. For SARS-CoV-2 diagnoses, serological investigations offer an alternative to RT–PCR. When real-time PCR and serological tests are combined, the rate of positive virus detection rises significantly. Total antibodies are the most sensitive and early serological marker, with levels rising as early as the second week after symptom start [34]. In most people, IgM levels rise during the first week following SARS-CoV-2 infection, peak after two weeks, and subsequently decrease to near background levels. After one week, IgG is

visible and remains at a high level for a long time [35].

During the severe acute respiratory syndrome (SARS) pandemic, various publications demonstrated that the detection of virus specific IgM and IgG are valid for serological diagnosis. According to studies, COVID-19 serodiagnosis based on IgM and IgG ELISA has a high sensitivity and specificity for the diagnosis of COVID-19.

The lateral flow assay is commonly employed in rapid point-of-care immunoassays. Rapid serological testing has two main approaches. First, identifying SARS-CoV-2 antigens, and second, determining anti-SARS-CoV-2 antibodies [36]. Immunological testing may play a key role in identifying those who have previously recovered from SARS-CoV-2 infection. The results of the test may also aid in the selection of convalescent plasma as a therapy option for COVID-19 patients.

E. Rapid antigen assay

A fast diagnostic test was developed to identify the presence of viral antigens produced by SARS-CoV-2 in samples from infected people' respiratory tracts. Antigen in the sample binds to antibodies attached to a paper strip encased in a plastic container for this test. Within half an hour, this reaction produces a clearly visible signal. Because the antigen(s) identified are only produced when the virus is actively replicating, the tests can be used to diagnose acute or early infection [37].

F. Viral culture

For viral isolation, nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage (BAL) fluid, sputum, and stool samples are obtained from suspected patients. A VeroE6 cell line expressing TMPRSS2 is extremely sensitive to SARS-CoV-2 infection, making it excellent for viral isolation and cytopathic testing. However, viral isolation and visualization are impracticable for large scale COVID-19 diagnosis since SARS-CoV-2 takes at least three days to inflict apparent cytopathogenic effects on chosen cell lines. Furthermore, because such facilities are not accessible

in the majority of health-care settings, virus isolation by cell culture is not advised for SARS-CoV-2 diagnostic purposes.

G. Radiographic testing

Combinations of radiographic, molecular and antigen-based assays have been used alone or in combination to determine the optimal means to make a definitive diagnosis of SARS-CoV-2 infection. Such examinations include a chest X-ray, CT or lung ultrasound. These, alone or together, can be also be used to stage SARS-CoV-2 infection. These chest x-ray abnormalities include bilateral lower zone and peripherally predominant consolidation and hazy opacities [38]. In addition, CT scans demonstrate a ‘reversed halo’ pattern and signs of septal thickening. Distinctive CT images illustrate bilateral pulmonary parenchymal ground glass and consolidated pulmonary opacities with occasionally rounded morphology and marginal lung dispersal [39].

III. Overview of SARS-CoV-2 risk factors

Assessment of key risk factors that predict disease course may be extremely useful for healthcare professionals in efficiently triaging patients, personalizing treatment, monitoring clinical progress, and allocating appropriate resources at all levels of care to reduce morbidity and mortality. Risk factors range from demographic factors such as age, gender, ethinicity, nutrition and life style habits to underlying diseases, complications, and laboratory indications [40].

A. Demographic factors

i. Older age and male gender

Older age is a major predictor of mortality and it is thus considered a key factor in the proposed clinical severity risk scores [40]. There was an unambiguous association between each stage of disease severity and sex with men having a higher risk of infection, disease severity, ICU admission and death than women [41].

ii. Ethinicity

When compared to participants of White ethnicity, Black and South Asian patients were shown to have a greater mortality risk [42]. Ethnic minority groups in England, such as Black ethnicity and Asian ethnicity, had a greater incidence of COVID-19 hospitalization, in the UK Biobankcohort of almost 400,000 people [43]. After accounting for socioeconomic, lifestyle, and health-related characteristics, the found relationships were weakened but remained significant.

B. Comorbidities

i. Arterial hypertension

Compared to nonsevere COVID-19 patients, arterial hypertension was seen more frequently in severe COVID-19 patients. According to one study, the incidence of hypertension was considerably greater among COVID-19 patients who required ICU treatment compared to those

who were not admitted to the ICU [8]. However, because hypertension is common in the elderly, this confounding factor should be ruled out.

ii. Diabetes

Diabetes is a prevalent co-morbidity that has been linked to a higher risk of severe and deadly COVID-19 complications. In type 2 diabetes mellitus patients, the expression of ACE2, the SARS-CoV-2 entry receptor, is elevated in the lungs and other organs. This increase is linked to chronic inflammation, endothelial cell activation, and insulin resistance, all of which exacerbate the inflammatory response and cause the alveolar-capillary barrier to become dysfunctional [44].

iii. Obesity

Body mass index (BMI) of greater than 40 kg/m^2 was found to be an independent risk factor for death, with the effect being stronger in individuals under the age of 50 [45]. After adjusting for age, sex, smoking, diabetes, hypertension, and dyslipidemia, obese COVID-19 patients with metabolic-associated fatty liver disease had a greater risk of catastrophic outcome [46].

iv. Chronic obstructive pulmonary disease (COPD)

COPD is not a predisposing factor for SARS-CoV-2 infection, according to a recent study, but once the patients develop the illness there is a higher risk of hospitalization, ICU admission, and invasive mechanical ventilation [47].

v. Chronic liver disease and chornic kidney disease (CLD and CKD)

Patients with CLD (cirrhosis, chronic hepatitis B, alcoholic liver disease, and other types of chronic hepatitis) are more vulnerable to infection due to altered immune function and are more likely to develop decompensation or acute-on-chronic liver failure from bacterial, fungal, or viral infection [48]. It has been found that individuals with coexisting CKD are at a greater risk of death than those who do not have CKD, and this is especially true at the end stage of CKD [42].

C. Laboratory Indicators

i. Leukocyte count

Viral infection causes dynamic changes in the numbers and subsets of peripheral blood leukocytes. Leukocytosis, or high leukocyte counts (9.5 x 10^9/L), was related with COVID-19 illness course, and the rise was more significant in severe and critically sick patients compared to nonsevere patients, suggesting that severe patients acquired more prominent inflammation [49].

ii. Platelet count

Low platelet counts were common in COVID-19 patients, particularly in the severely and critically sick. Reduced platelet generation, accelerated platelet breakdown, and consumption, as in other viral infections, may all contribute to thrombocytopenia [50].

iii. D-dimer

Elevated D-dimer levels are prevalent in COVID-19 patients and may be related to sepsis-induced coagulopathy, as well as reflecting the increased thromboembolic risk in severe COVID-19 cases [51].

iv. C-reactive protein

High levels of serum CRP are important indications of illness progression and a risk factor for mortality in COVID-19 patients, and they are symptomatic of a growing cytokine storm in COVID-19 patients [52]. CRP levels increased generally in the first seven days after ICU admission, peaking between days two and three, according to laboratory examination of patients admitted to the ICU [53].

v. IL-6

SARS-CoV-2 can activate the pyrin domain carrying 3-inflammasome in monocytes/macrophages, leading to the production of high quantities of proinflammatory mediators such as IL-6 and IL-1, as well as increased cell death and a cytokine strom [53].

vi. Type I interferon (IFN-I)

IFN-I is essential for viral immunity and a healthy immune system. It has been proposed that

the IFN-I response contributes to severe illness. COVID-19, a severe and dangerous illness [54]. Due to hyperinflammation, COVID-19 patients showed reduced IFN-I activity and strong inflammatory gene expression in blood cells or BAL fluid macrophages [55].

vii. Ferritin

Serum ferritin levels were linked to death and the development of severe outcomes in COVID-19. Multiorgan failure and hyperferritinemia can occur as a result of cytokine storm syndrome [56].

D. Diet and lifestyle

i. Vitamin C and vitamin D

Vitamin C functions as an antioxidant and cofactor for regulatory enzymes, as well as influencing both the innate and adaptive immune systems [57]. Vitamin C has recently been shown to inhibit proinflammatory and procoagulant pathways, therefore ameliorating vascular and lung damage in sepsis and ARDS [58].

It is generally known that vitamin D regulates gene transcription and immunological response. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25-$(OH)_2$ D_3), controls nuclear factor (NF)-B activity and then causes the creation of several molecules that enhance the inflammatory response, including IL-6, IL-1, TNF-α, and IFN-γ. According to a recent analysis, vitamin D improves the inflammatory response via several pathways, protects against respiratory infections, and lowers the risk of influenza and COVID-19 associated pneumonia [59]. Vitamin D has two principal metabolites: vitamin D3 and vitamin D2. When exposed to UV-B light, unstable 7-dehydrocholesterol in the skin is converted to pre-vitamin D3 and stable vitamin D3, respectively. Vitamin D3, also known as cholecalciferol, is found in dairy products, eggs, and fish. During the hydroxylation process in the liver, vitamin D3 is transformed to 25-hydroxyvitamin D3 (25(OH) D3) by the 25-hydroxylase enzyme. The 25(OH) D3 form is subsequently transferred to the kidney, where it is converted to 1,25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D, via 1-

α hydroxylase [60].

IV. Methods

A. Patient and data source

A positive case was classified as one who has COVID-19 verified by viral isolation and/or real-time reverse transcription-PCR (rRT-PCR) targeting the in-house developed N-gene as detailed below, as well as employing the Kogene kit (Kogene Biotech Seoul, South Korea) to target the E and RdRP genes. Negative sera were acquired before the COVID-19 pandemic. The serial respiratory and serum samples were obtained from patients during their stay at Chosun University Hospital. To determine the antibody response, patients were also recruited from Yeungnam University Hospital and Seoul National University Bundang Hospital, Korea.

B. Viral RNA extraction

Using a Real-prep viral DNA/RNA kit, a fully automated device (BioSewoom South Korea) was employed to extract viral RNA (BioSewoom, South Korea). The extraction was carried out using 200 μL of all samples, according to the manufacturer's instructions, yielding a final elution of 100 μL. Following that, the samples were kept at -80°C until they were utilized for rRT-PCR analysis.

C. SARS-CoV-2 cell culture and detection of infectious virus

SARS-CoV-2 respiratory samples incubated in Vero E6 cells (Korean Cell Line Bank, KCLB no. 21587), using 6-well cell culture plates. For virus isolation, each clinical sample was treated with 20x penicillin/streptomycin at 4℃ for 1 h, then the supernatant was used to infect Vero E6 cells. The infected cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum and 1 × penicillin–streptomycin solution (Gibco, ThermoFisher Scientific Inc., USA) and then cultured at 37 °C under the presence of 5% CO_2 for 3–5 days by daily observing the cytopathic effect (CPE). Subsequently, viral proliferation was confirmed on the basis of a Ct value of < 20 using rRT-PCR

after two passages. The results were characterized as negative if no CPE were observed within 5 days. Furthermore, viral RNA was extracted using the culture supernatant and analyzed via rRT-PCR at two passages to validate the proliferation of SARS-CoV-2. The completed assay was performed in Bio-safety level-3 at Health and Environment Research Institute of Gwangju city.

D. Detection of SARS-CoV-2 RNA by one step quantitative RT-PCR (RT-qPCR)

The E-gene and RdRp gene were identified as per manufacturer's protocol (Kogene Biotech Seoul, South Korea). To target the N gene for detecting SARS-CoV-2, the primers and probe were designed in-house. Briefly, 5 µL of template was added to 4 µL of 5X RT-qPCR mixture (Roche), 0.5 µL of 200X RT enzyme solution (Roche), 1 µL (10 pmol/µL) of forward primer (nCov-NP-572F 5′-GCAACAGTTCAAGAAATTC-3′), 1 µL (10 pmol/µL) of reverse primer (nCov-NP-687R-5′-CTGGTTCAATCTGTCAAG- 3′), 1 µL (5 pmol/µL) of probe (nCov-NP-661P-5′-FAM-AAGCAAGAGCAGCATCACCG-BHQ1-3′), and 7.9 µL of RNAase free water to obtain a total reaction mixture of 20 µL. The analysis was performed in an Exicycler™ 96 (Ver.4) Real-Time Quantitative Thermal Block (Bioneer, South Korea) under the following cycle conditions: 1 cycle at 50 °C for 10 min and 95 °C for 30 s followed by 45 cycles at 95 °C for 5 s and 57 °C for 30 s. SARS-CoV-2 sgRNAs were identified via RT-PCR as previously described [61].

The cycle threshold value (Ct-value) was analyzed using the Bioneer Package software, and the sample was considered positive if a visible amplification plot was observed at $Ct \leq 35$ and negative with $Ct > 35$. In order to determine the viral load of N-gene, Ct-values were converted to Log_{10} RNA copies/mL by utilizing the calibration curves as previously described [61].

E. Identification of total antibody against rNP of SARS-CoV-2 by ELISA

Serum antibodies against SARS-CoV-2 were determined using an indirect ELISA. The 96-well ELISA microplates (Thermo Fisher Scientific Korea. Ltd.) were coated overnight at 4°C with 100 µL per well of 2 µg/mL of plant- and *E. coli*-expressed rNPs. The microplates were washed

three times with washing buffer of PBS-T (0.05% Tween 20) and blocked with blocking buffer (PBS-T containing 5% of skim milk) for 2 h at 37°C. After four washes, the specimens were diluted 100-fold with blocking solution and incubated at 37◦C for 2 h. The plates were then washed five times with washing buffer. Following this, HRP-conjugated goat anti-human total Ab (Thermo Fisher Scientific, Cat 31418) was diluted in blocking solution (1:40,000) and added at 100 μL volume per well and incubated at 37°C for 1 h. After extensive washing, 50 μL of 3,3′,5,5′-tetramethylbenzidine substrate (TMB; Sigma-Aldrich, USA) was added to each well at room temperature in the dark. After 30 min, the reaction was stopped with 25 μL of 1M H_2SO_4, and the absorbance at 450 nm was measured in each well. The samples were tested in triplicate.

F. Recombinant protein

The rNPs were produced and synthesized commercially using Nicotiana benthamiana plants (BioApplications Inc., Korea) and *E. coli* (Bionics, Korea). Both proteins had the same 420 amino acid sequence. Protein purity and antigenicity were confirmed by SDS-PAGE and western blotting, respectively.

G. SDS-PAGE and western blotting

To validate the antigenicity and protein purity of plant- and *E. coli*-based rNPs, we tested a serum sample positive for SARS-CoV-2 infection obtained from a patient in the convalescent phase. For negative control the pooled negative sera were obtained from the healthy individuals prior to the COVID-19 pandemic. The proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting of protein bands onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Germany). The blot was cut into strips and blocked with blocking buffer (phosphate-buffered saline with Tween 20 [PBS-T] containing 5% skimmed milk) for 1 h at room temperature, followed by incubation with serum samples as primary antibodies (1:1000 diluted in blocking buffer) at 4◦C overnight. After washing in PSB-T for 10 min,

the bound antibodies were detected using horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-human IgG (Invitrogen, USA) at a dilution of 1:10,000 in PBS-T for 1 h at room temperature. The immunoprecipitated bands were developed using enhanced chemiluminescence reagents, and the membranes were scanned with an infrared imaging system. The expression and purity of both proteins were identified by SDS-PAGE gel stained with InstantBlue® Coomassie Protein Stain (ab119211).

H. SD Biosensor STANDARD™ E COVID-19 Total Ab ELISA

The assay was performed according to the manufacturer's instructions (Catalogue no; E-NCOV-01T, SD Biosensor, Inc. South Korea). The assay was intended to detect total antibodies (IgM/IgA/IgG) to SARS-CoV-2 in human serum by binding to the pre-coated spike protein on the microplate. The cut-off value was calculated by adding the mean absorbance at 450 nm of the negative control to 0.3.

I. EDI™ Novel Coronavirus COVID-19 IgG and IgM ELISA

The assay was performed according to the manufacturer's protocol (Epitope Diagnostic, Inc. San Diego, CA 92121, US). The ELISA assay detects IgG (Catalogue no; KT-1032) specific antibodies in human serum by binding to SARS-CoV-2 recombinant full-length nucleocapsid protein coated on the plates. The ELISA assay that detects IgM-specific antibodies (Catalogue no; KT-1033) is based on the capture of IgM in human serum and then detects antibodies binding to SARS-CoV-2 nucleocapsid protein. The cut-off values were calculated by adding the average OD of negative controls to 0.18 (for IgG) or 0.10 (for IgM) and multiplying by 0.9 and 1.1 to obtain the negative and positive results, respectively.

J. Detection of SARS-CoV-2 by Antigen-Detection Rapid Diagnostic Tests (Ag-RDTs)

The samples were tested with two lateral flow assays: PCL COVID19 Ag Rapid FIA (fluorescence immunoassay) and PCL COVID-19 Ag Gold (PCL, Inc. South Korea); both are diagnostic medical devices that use a dual antibody sandwich reaction with an immunochromatographic assay to quantitatively detect the N-antigen of SARS-CoV-2 in human respiratory specimens. The recommended instructions for use according to the manufacturer include incorporation of the sample into the extraction buffer; however, we analyzed the samples in VTM, since it enabled rapid assessment of numerous previously characterized rRT-PCR clinical samples. Based on this approach, the manufacturer instructed the application of 100 µL of the sample immediately into the test card. Prior to testing, the samples were thawed and kept at room temperature. The samples were then vortexed and transferred into the test card well with an average incubation time of 15 min at room temperature. For Ag Rapid FIA, results were observed with the PCL OK EZ automated analyzer, in the quick test mode. Thereafter, the results of Ag Gold were read visually and recognized by two different individuals, who mutually decided the final result. The complete assay was performed in a Bio-safety level-2 facility with full personal protective equipment.

K. Classification of vitamin D [25(OH) D] levels

Vitamin D sufficiency is commonly assessed by measuring total serum 25(OH) D concentration, defined as the sum of 25-hydroxyvitamin D_3 and 25(OH) D_2 levels [62]. The serum concentration of other vitamin D metabolites are measured in certain conditions. For instance, levels of $1,25(OH)_2$ D were measured if suspecting a defect in conversion of 25(OH) D to $1,25(OH)_2$ D as found in hypoparathyroidism, renal failure, or osteomalacia [63]. The levels of vitamin D were classified as normal, insufficient and deficient with serum 25 (OH) D levels of > 30 ng/mL, 20-30ng/mL and < 20ng/mL, respectively [64].

L. Classification of patient severity

Based on the degree of severity the patients were classified into four groups. CDC criteria were used for the classification of disease severity. As per guidelines, the classification of the patients was based on the following clinical parameters:

(1) Asymptomatic case: Individuals tested positive for SARS-CoV-2 but did not have any symptoms of COVID-19.

(2) Mild to moderate case: Individuals with presence of clinical symptoms including fever, cough, or change in taste or smell, dyspnea and/or hypoxia, evidence of lower respiratory disease on clinical assessment or imaging and an oxygen saturation (SpO2) of $\geq$ 94% on room air.

(3) Severe case: Individuals with clinical signs of pneumonia with one of the following: SpO2 of < 94% on room air, respiratory rate of $\geq$ 30 breaths/min, and lung infiltrates of > 50%.

(4) Critical case: Individuals requires mechanical ventilation with clinical signs of respiratory failure, septic shock, and/or multiorgan dysfunction or failure.

M. Statistical analysis

Sensitivity was defined as the percentage of patients accurately detected as having COVID-19, as initially diagnosed using rRT-PCR and SARS-CoV-2 culture from respiratory specimens. Specificity was defined as the percentage of patients who were accurately identified as not having SARS-CoV-2 infection. The categorical variables were recorded as percentages and counts with Wilson score at 95% confidence intervals (CI), whereas the continuous variables were presented as mean, standard deviation (SD) or median, and interquartile range (IQR). The differences between means were compared using two sample t-tests. The McNemar test was used to analyze the test differences in dependent groups. The normality was evaluated using Kolmorgorov–Smirnov test. To compare the mean between two groups, the analysis was performed using two sample t-tests;

for more than two groups, one-way analysis of variance (ANOVA) was used. To identify the effect of Vitamin D on 30-day survival time, a Kaplan-Meier survival analysis was conducted. To determine the accuracy of the assay, the receiver operating characteristic (ROC) curve was generated, and area under the ROC curve (AUC) was observed [65]. To determine the best cut-off value for the ELISA assays, we implemented two different calculation methods:

(A) The first optimal cut-off value (maximum trade-off between sensitivity and specificity) was identified by generating ROC curve.

B) For in-house total Ab ELISA, the second cut-off value was identified by OD_{450} plus 3-fold standard deviation (mean +3SD) by utilizing negative controls. For SD Biosensor-Total Ab ELISA and for EDI-IgG and IgM ELISA, the cut-off values were calculated using the manufacturer's method. All data were analyzed using MedCalc statistical software (Ostend, Belgium), and the *p*-values were reported as two tailed with < 0.05 indicating statistical significance.

VI. Results

A. Clinical performance of in-house and commercial ELSIA assays

i. Patients and source of data

For this analysis, we recruited patients between February 2020 and July 2020. For both rNP-based and SD Biosensor ELISAs, 141 serial samples were obtained from 32 recruited patients who were positive for SARS-CoV-2 infection. To compare the performance of in-house total Ab ELISA with the EDI-ELISA assay, a total of 51 samples from 21 SARS-CoV-2 confirmed patients were used as positive controls, while 20 samples were used as negative controls.

ii. Specificity

The specificity varied between 94.30% (95% CI; 89.46-97.36) and 98.10% (95% CI; 94.55 99.61) for the plant rNP- and *E. coli* rNP-based assays, respectively (p = 0.109). Two samples were false positive for SD Biosensor with a specificity of 98.73% (95.50. 99.85). The specificity was 100% (95% CI; 83.16, 100) and 80% (95% CI; 56.34, 94.27) for EDI-IgG and IgM ELISA, respectively (p = 0.125) (Table 1)

iii. Sensitivity

The overall sensitivity varied between 92.91% (95% CI; 87.34, 96.55) for plant rNP, 83.69% (95% CI; 76.54, 89.37) for SD Biosensor, and 75.89% (95% CI; 67.97, 82.69) for *E. coli* rNP (Table 1). All false negatives of the plant rNP-based and SD Biosensor ELISA were obtained from samples obtained within 10 days post-symptom onset (PSO) (Figure 3 A and C). The sensitivity of the EDI-ELISA assay was calculated using 51 samples. The overall sensitivity of EDI-IgM ELISA was 80.39% (95% CI; 66.88, 90.18), which was slightly greater than that observed for EDI-IgG ELISA at 76.47% (95% CI; 62.51, 87.21) (p = 0.8145). Among all the assays, the plant rNP-based ELISA

showed the highest sensitivity (92.91%) and accuracy (93.65% CI; 90.25, 96.13), with a statistically significant difference ($p < 0.05$).

Table 1 : Performance of in-house rNP Plant-and *E. coli*-based, SD Biosensor-Total Ab, and EDI™ Novel Coronavirus COVID-19 IgG and IgM ELISA

Methods		rNP (Plant-based)	rNP (*E. coli*-based)	SD Biosensor-Total Ab	EDI-IgG	EDI-IgM
ROC curve	Cut-off	0.5	1.8	0.18	0.28	0.09
	True Positive	131/141	107/141	118/141	39/51	41/51
	True Negative	149/158	155/158	156/158	20/20	16/20
	Intermediate	N/A	N/A	N/A	N/A	N/A
	Sensitivity (%) (95% CI)	92.91 (87.34, 96.55)[a]	75.89 (67.97, 82.69)	83.69 (76.54, 89.37)	76.47 (62.51, 87.21)	80.39 (66.88, 90.18)
	Specificity (%) (95% CI)	94.30 (89.46, 97.36)	98.10 (94.55, 99.61)	98.73 (95.50, 99.85)	100 (83.16, 100)	80 (56.34, 94.27)
	Accuracy (%) (95% CI)	93.65 (90.25, 96.13)	87.63 (83.35, 91.14)	91.64 (87.90, 94.52)	83.10 (72.34, 90.95)	80.28 (69.14, 88.78)
	AUC	0.980 (0.957, 0.993)[a]	0.929 (0.893, 0.955)	0.923 (0.887, 0.951)	0.894 (0.798, 0.955)	0.805 (0.694-0.890)
Mean +3SD	Cut-off	0.7	2.2	0.36	0.32 - 0.39	0.18 - 0.22

or range/value by commercial assay	True Positive	118/141	90/141	115/141	37/51	24/51
	True Negative	158/158	158/158	157/158	20/20	19/20
	Intermediate	N/A	N/A	N/A	2/71	1/71
	Sensitivity (%) (95% CI)	83.69 (76.54, 89.37)[b]	63.83 (55.32, 71.75)	81.56% (74.16, 87.59)	72.55 (58.26, 84.11)[c]	45.28 (31.56, 59.55)
	Specificity (%) (95% CI)	100 (97.69, 100)	100 (97.69, 100)	99.37 (96.52, 99.98)	100 (83.16, 100)	95 (75.13, 99.87)
	Accuracy (%) (95% CI)	92.31 (88.68, 95.06)	82.94 (78.19, 87.03)	90.97 (87.13, 93.96)	80.28 (69.14, 88.78)	58.90 (46.77, 70.29)

ROC, receiver operating characteristic; 95% CI, 95% confidence interval

[a] $p < 0.05$ vs all

[b] $p < 0.05$, all except SD Biosensor-Total Ab

[c] $p < 0.05$, vs. EDI-IgM.

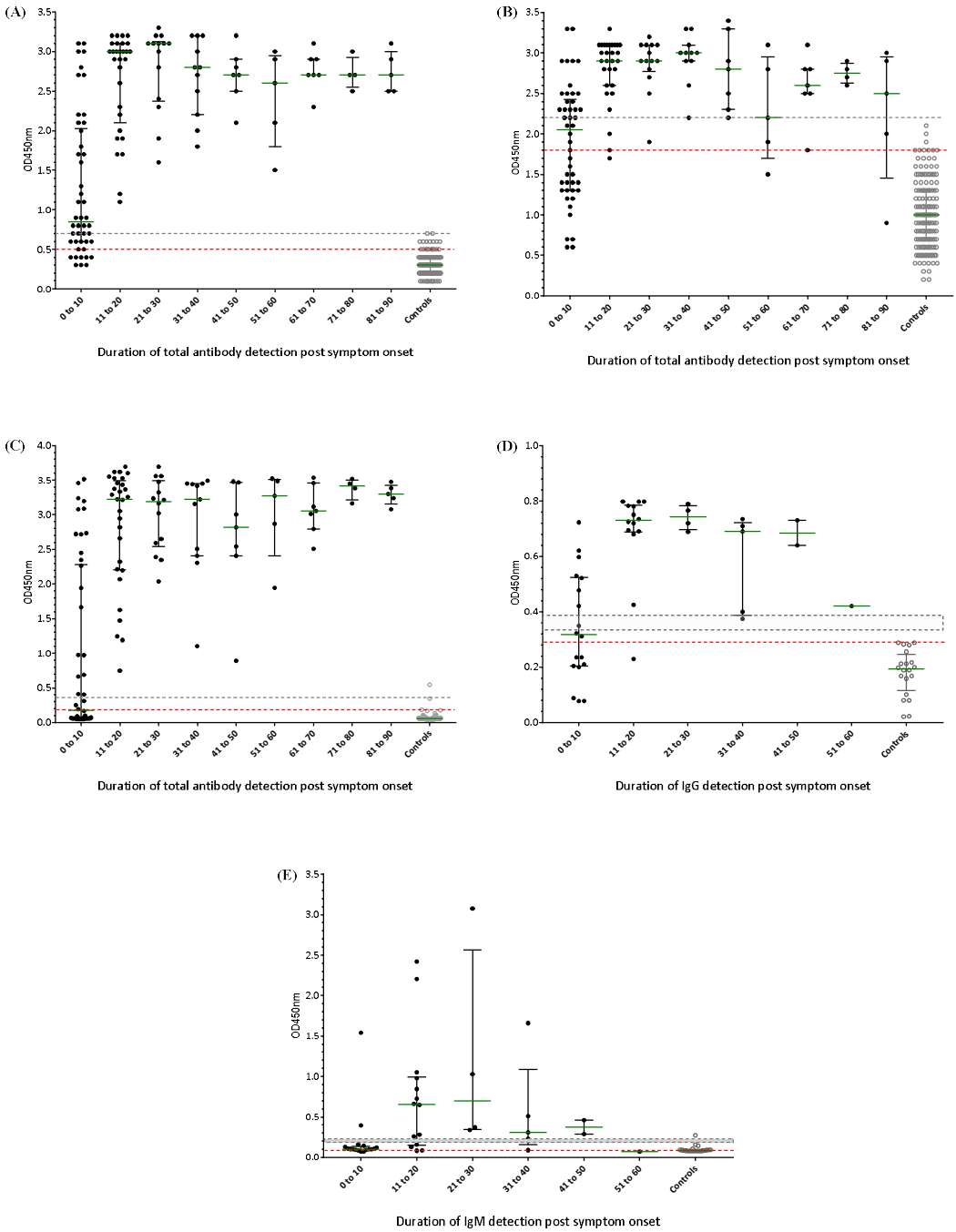
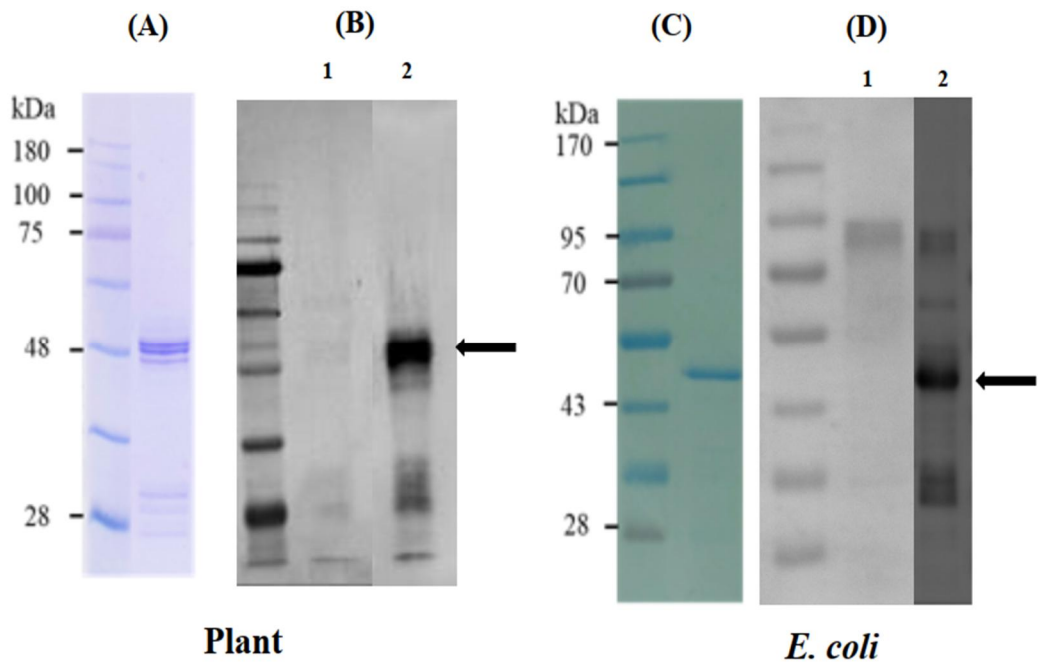


Figure 3: Optical density at 450 nm (OD_{450}) for antibody detection by days after symptom onset.

(A) Plant-based rNP **(B)** *E. coli*-based rNP **(C)** SD Biosensor-Total Ab **(D)** EDI – IgG and (E) EDI – IgM ELISA assays. The grey line shows the calculated cut-off values by mean + 3SD or range recommended by commercial assay. The red line shows the cut-off values recommended by ROC curve. Green lines indicate median with interquartile ranges.

iv. SARS-CoV-2 antigen expression in plant and *E. coli*

The purity of plant- and *E. coli*-based rNPs was assessed by SDS-PAGE and Coomassie-blue staining (Figure 4 A and C). The antigenicity of the recombinant proteins was evaluated by western blotting using patient's serum sample (Figure 4 B and D). The results showed the expected



molecular weights of ~48 kDa and ~46 kDa for plant- and *E. coli*-based rNP, respectively.

Figure 4: Analysis of plant- and *E. coli*-based SARS-CoV-2 recombinant nucleocapsid protein (rNP)

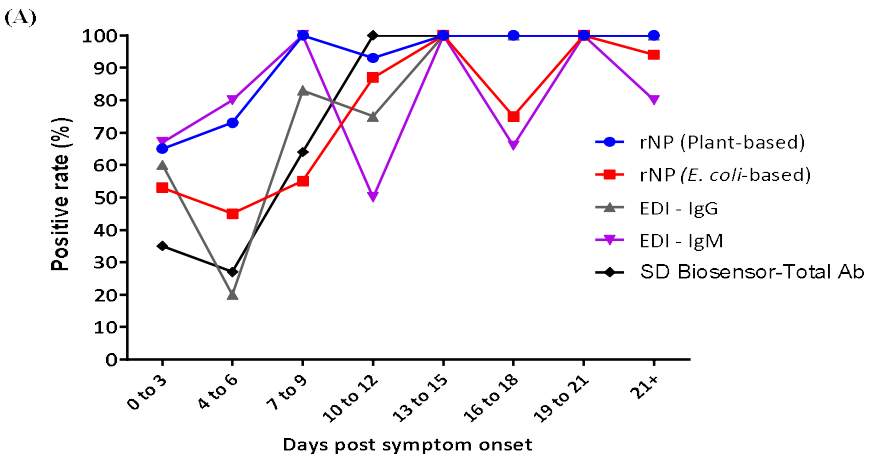
Lines on the left indicated the molecular weight marker (protein ladder) in kDa. The arrows indicates the expected sizes for recombinant N proteins. Lanes 1A and 1B were blotted with pooled negative sera, while Lanes 2A and 2B with serum samples positive for SARS-CoV-2 infection obtained from a patient in the convalescent phase.

v. Dynamic trend to seropositivity against SARS-CoV-2 relative to the duration of illness

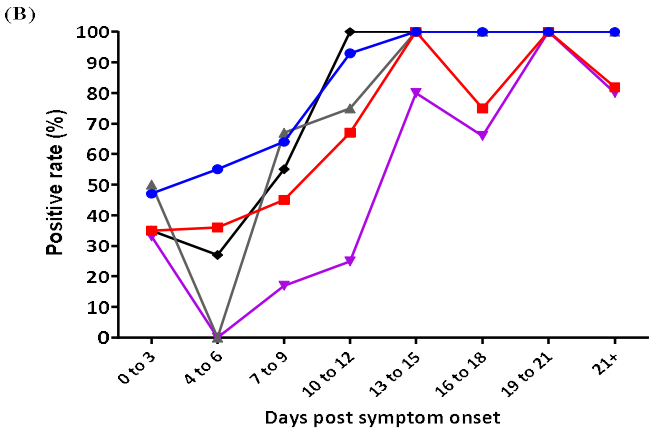
The sensitivity of EDI-IgM was highest in week 1 at 72.73% (95% CI; 39.03-93.98), followed by plant rNPs at 67.86% (95% CI; 47.65-84.12). The sensitivity of all assays increased during week 2 (Table 2). For plant rNPs, SD Biosensor, and EDI-IgG ELISA, the seroconverted samples reached a plateau at 100% 10 and 12 days PSO (Figure 3 A, C, D, and Figure 5). None of the patients became seronegative after the first positive result obtained with plant rNPs, SD Biosensor, and EDI-IgG assays; however, no such pattern was observed in the samples tested with *E. coli* rNPs and EDI-IgM ELISA assays (Figure 5). The seroconversion of total Ab was observed to occur as early as the day of symptom onset, with a median of 5 days PSO (interquartile range [IQR], 1-9 days) for plant rNPs, 7 days PSO (IQR, 1-10 days) for *E. coli* rNP, and 8 days PSO (IQR, 5-10 days) for SD Biosensor ELISA. For EDI-IgG and-IgM ELISA assays, the day of seropositivity could not be identified as complete serial samples were not analyzed using these kits.

vi. ROC analysis

The AUC obtained from ROC analysis provides a good parameter for the diagnostic power of a specific test and was compared among the different ELISA assays (Figure 6). Compared to all assays, the plant-based rNPs had the significantly highest measure at 0.957 (95% CI; 0.881-0.991, $p < 0.05$).



	Days	0-3	4-6	7-9	10-12	13-15	16-18	19-21	21+
Total antibody	Number	(N = 17)	(N = 11)	(N = 11)	(N = 15)	(N = 10)	(N = 8)	(N = 6)	(N = 50)
	rNP (Plant-based)	11	8	11	14	10	8	6	50
	rNP (*E. coli* -based)	9	5	6	13	10	6	6	47
	SD Biosensor	6	3	7	15	10	8	6	50
EDI - ELISA	Number	(N = 6)	(N = 5)	(N = 6)	(N = 4)	(N = 5)	(N = 3)	(N = 5)	(N = 10)
	IgG	3	1	5	3	5	3	5	10
	IgM	4	4	6	2	5	2	5	8



	Days	0-3	4-6	7-9	10-12	13-15	16-18	19-21	21+
Total antibody	Number	(N = 17)	(N = 11)	(N = 11)	(N = 15)	(N = 10)	(N = 8)	(N = 6)	(N = 50)
	rNP (Plant-based)	8	6	7	14	10	8	6	50
	rNP (*E. coli* -based)	6	4	5	10	10	6	6	41
	SD Biosensor	6	3	6	15	10	8	6	50
EDI-ELISA	Number	(N = 6)	(N = 5)	(N = 6)	(N = 4)	(N = 5)	(N = 3)	(N = 5)	(N = 10)
	IgG	3	0	4	3	5	3	5	10
	IgM	2	0	1	1	4	2	5	8

Figure 5: Graph of the positive rate of SARS-CoV-2-specific total antibodies, IgG, and IgM by days post-symptom onset.

(A) Positive rate obtained from recommended cut-off values by ROC curve **(B)** Positive rate obtained from calculated cut-off values by mean + 3SD or range recommended by commercial assay.

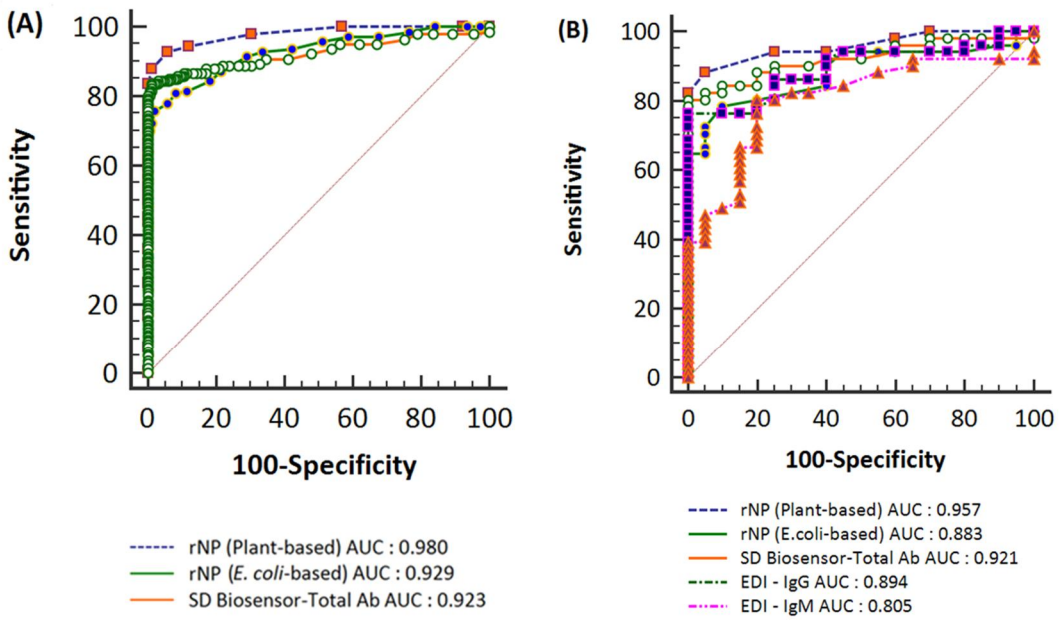


Figure 6: Receiver operating characteristic (ROC) curves for the evaluation and comparison of diagnostic accuracy

(A) Plant rNP-, *E. coli* rNP-based and SD Biosensor-Total Ab ELISA assays and **(B)** Plant rNP-, *E. coli* rNP-based, SD Biosensor-Total Ab, EDI-IgG and IgM ELISA assays

Table 2: Sensitivity of in-house rNP Plant-based, *E. coli*-based, SD Biosensor-Total Ab, and EDI^{TM} Novel Coronavirus COVID-19 IgG and IgM ELISA assays with the duration of illness

Sensitivity (%) (Number, 95%CI)	rNP (Plant-based)	rNP (*E. coli*-based)	SD biosensor-Total Ab	EDI-IgG	EDI-IgM
	ROC curve				
Week 1	67.86 (19/28, 47.65-84.12)	50.00 (14/28, 30.65-69.35)	67.86 (19/28, 47.65-84.12)	36.36 (4/11, 10.93-69.21)	72.73 (8/11, 39.03-93.98)
Week 2	96.15 (25/26, 80.36-99.90)	69.23 (18/26, 48.21-85.67)	84.62 (22/26, 65.13-95.64)	80.00 (8/10, 44.39-97.48)	80.00 (8/10, 44.39-97.48)
Week 3	100 (21/21, 83.89-100)	95.24 (20/21, 76.18-99.88)	100 (21/21, 83.89-100)	100 (11/11, 71.51-100)	90.91 (10/11, 58.72-99.77)
> Week 3	100 (53/53, 93.40-100)	94.34 (50/53, 84.34, 98.82)	100 (53/53, 93.40-100)	100 (12/12, 73.54-100)	83.33 (10/12, 51.59-97.91)
	Mean + 3SD or range/value by commercial assays				
Week 1	50.00 (14/28, 30.65-69.35)	35.71 (10/28, 18.64-55.93)	32.14 (9/28, 15.88-52.35)	27.27 (3/11, 6.02-60.97)	18.18 (2/11, 2.28-51.78)

Week 2	88.77 (21/26, 60.65-93.45)	53.85 (14/26, 33.37-73.41)	73.08 (19/26, 52.21-88.43)	70.00 (7/10, 34.75-93.33)	20.00 (2/10, 2.52-55.61)
Week 3	100 (21/21, 83.89-100)	95.24 (20/21, 76.18-99.88)	100 (21/21, 83.89-100)	100 (11/11, 71.51-100)	81.82 (9/11, 48.22-97.72)
> Week 3	100 (53/53, 93.28-100)	83.02 (44/53, 70.20-91.93)	100 (53/53, 93.28-100)	100 (12/12, 73.54-100)	83.33 (10/12, 51.59-97.91)

ROC, receiver operating characteristic; mean + 3SD, mean optical density plus 3 fold standard deviation; 95% CI, 95% confidence interval.

Week 1 (0–6 days PSO); Week 2 (7–13 days PSO); Week 3 (14–20 days PSO); > Week 3 (≥ 21 to 91 days PSO).

B. Clinical performance Antigen-Detection Rapid Diagnostic Tests (Ag-RDTs)

i. Patients and source of data

Between February 2020 and August 2020 we performed a cohort study of patients with confirmed COVID-19. A total of 150 samples were tested; of these, 63 serial samples were obtained from SARS-CoV-2 positive patients [oropharyngeal swab (n = 10), nasopharyngeal swab (n = 26), and saliva (n = 27)] and 87 samples from healthy individuals as negative controls. All 63 samples were tested via rRT-PCR, cell culture, and Ag rapid FIA; however, only 54 samples were available for Ag Gold analysis. The samples included in the present study were obtained 2 days prior to the symptom onset (−2) up to 25 days postsymptom onset (PSO). Most samples were collected during the early stage of disease course with median duration of 1 day PSO (IQR; −1.25 to 5.25). Of the 63 samples, 51 (80.59%) were RT-PCR positive with mean Ct-value of 26.52 (±4.74; range, 16.56–34.47) equivalent to 6.5 $\log_{10}$ RNA copies/mL (range, 9.28–4.41 $\log_{10}$ RNA copies/mL).

ii. Ag-RDT performance in correlation with rRT-PCR

Overall, for rRT-PCR positive samples, the detection sensitivity of Ag rapid FIA (n = 51) and Ag Gold (n = 43) was 74.51% (38/51, 95% CI; 60.4–85.7) and 53.49% (23/43, 95% CI; 37.7–68.8), respectively. Both assays were performed with 100% specificity for rRT-PCR negative samples (n = 99 and n = 98). Discordant results (rRT-PCR +ve/Ag-RDT −ve) were observed in 13 samples for Ag rapid FIA and in 20 samples for Ag Gold (Figure 7). The ROC-curve analysis revealed an AUC of 0.837 (95% CI; 0.808–0.921) and 0.767 (95% CI; 0.689–0.834), respectively ($p < 0.001$) (Figure 8). According to the range of mean Ct-values (≤20, > 20 – ≤ 25, > 25 – ≤ 30, > 30 – ≤ 35, > 35),

both antigen assays revealed 100% detection rate (95% CI; 47.82–100) for samples at Ct < 20. A noticeable decrease in the detection rate of Ag Gold was observed at Ct > 25, with overall sensitivity of 80% (95% CI; 56.34–94.27) at Ct ≤ 25; however, Ag rapid FIA sustained detection rate of 82.61% (95% CI; 68.58–92.18) until Ct ≤ 30. No detection was observed at Ct > 35 for both Ag-RDTs (Table 3). Furthermore, in terms of days PSO, the sensitivities of Ag-RDTs are higher at the initial stage of the disease course, followed by a progressive decline in further ranks (Table 4).

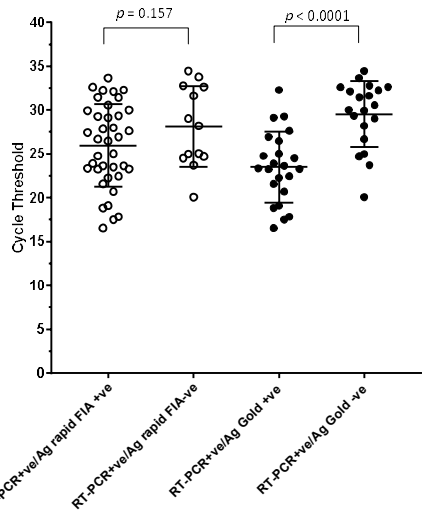


Figure 7: rRT-PCR cycle threshold values (N-gene) in samples testing either Ag-RDT positive or negative.

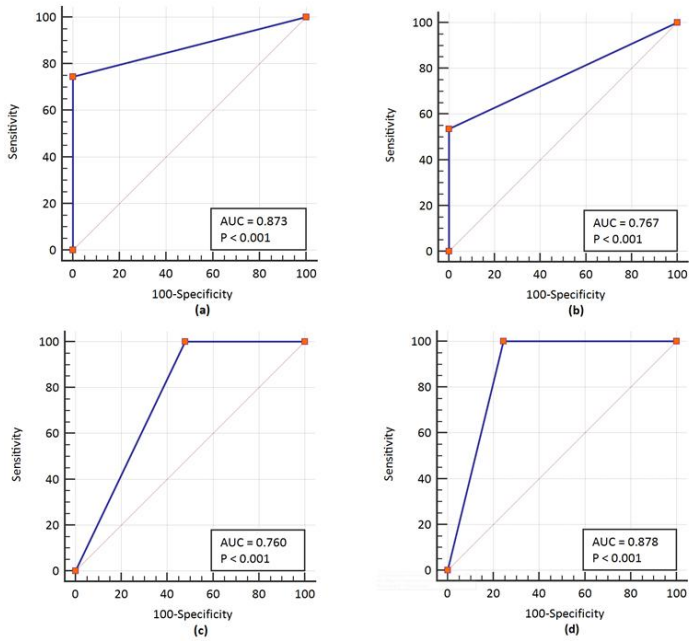


Figure 8: ROC-curve analysis with respect to the two diagnostic classification variables

By using rRT-PCR as a reference, **(a)** Ag rapid FIA and **(b)** Ag Gold. Using cell culture test as a reference, **(c)** Ag rapid FIA and **(d)** Ag Gold.

Table 3: Sensitivity of Ag rapid FIA and Ag Gold assays determined by SARS-CoV-2 N gene rRT-PCR cycle threshold (Ct) value

Ct-value	*N*	Positive	Negative	Sensitivity(%)	95% CI (%)
Ag rapid FIA					
≤ 20	5	5	0	100.00	47.82–100
> 20–≤ 25	17	12	5	70.59	44.04–89.69
> 25–≤ 30	16	13	3	81.25	54.35–95.95
> 30–≤ 35	13	8	5	61.54	31.58–86.14
> 35	12	0	12	0.00	0.00–26.46
Ag Gold					
≤ 20	5	5	0	100.00	47.82–100
> 20–≤ 25	15	11	4	73.33	44.90–92.21
> 25–≤ 30	12	6	6	50	21.09–78.91
> 30–≤ 35	11	1	10	9.09	0.23–41.28
> 35	11	0	11	0	0.00–28.49

rRT-PCR; real time reverse transcription polymerase chain reaction, CI; confidence interval, N; number of total samples

Table 4: Sensitivity of Ag rapid FIA and Ag Gold determined by days post symptom onset

Days post symptom onset	Ct-value (mean ± SD)	*N*	Positive	Negative	Sensitivity (%)	95% CI (%)
Ag rapid FIA						
−2–3	24.94 ± 5.72	21	15	6	71.43	47.82–88.72
4–7	27.47 ± 4.45	24	15	9	62.50	40.59–81.20
8–14	33.41 ± 3.78	13	6	7	46.15	19.22–74.87
>14	34.9 ± 2.86	5	2	3	40.00	5.27–85.34
Ag Gold						
−2–3	24.52 ± 5.80	18	14	4	77.78	52.36–93.59
4–7	27.41 ± 4.86	19	7	12	36.84	16.29–61.64
8–14	33.25 ± 3.90	12	2	10	16.67	2.09–48.41
>14	34.90 ± 2.86	5	0	5	0.00	0.00–52.18

CI; confidence interval, N; number of total samples

iii. Ag-RDT performance in correlation with *in vitro* infection and sgRNA

We further evaluated the performance of Ag-RDTs with respect to infectious SARS-CoV-2 samples and sgRNA. The analysis revealed that both Ag rapid FIA and Ag Gold detected 100% (95% CI; 78.20–100 and 75.29–100) of the infectious samples (15/15 and 13/13) and showed better performance in distinguishing infectious samples compared to sgRNA PCR assay [66.66% (10/15), $p = 0.06$ and 0.25] (Figure 9). The ROC-curve analysis revealed an AUC of 0.760 (95% CI; 0.636–0.859) and 0.878 (95% CI; 0.760–0.951) for Ag rapid FIA and Ag Gold, respectively ($p < 0.001$) (Figure 3). Only 29.4% (15/51) of rRT-PCR positive samples were infectious when tested by cell culture infectivity. In contrast, both Ag-RDTs demonstrated better correlation with cell culture infectivity [39.47% (15/38, $p = 0.322$) and 56.52% (13/23, $p = 0.027$)]. For both Ag-RDTs, all these samples yielded discordant results (rRT-PCR +ve/Ag-RDT −ve) and were detected as negative on culturing. Furthermore, the data suggest that detection of sgRNA is not well correlated with that of infectious virus in Vero E6 cells and was predicted poorly if cell cultures were positive (PPV of 47.62%, 95% CI, 32.62–63.06) (Figure 9).

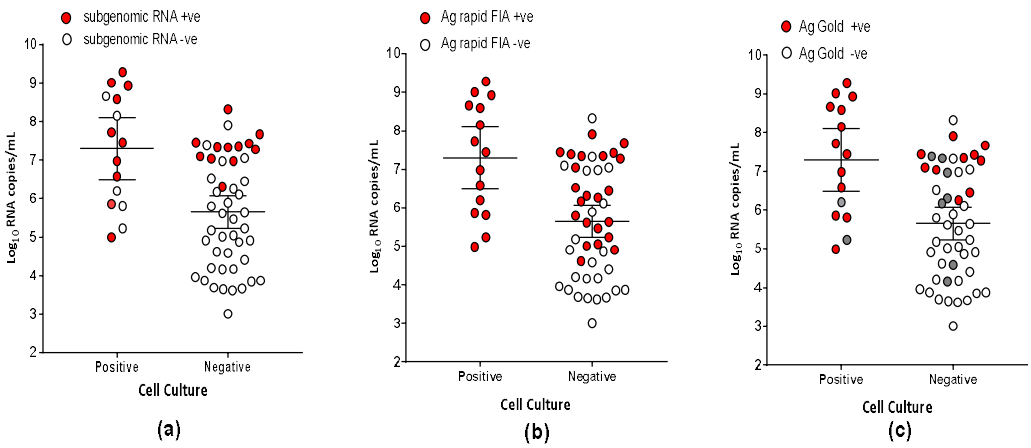


Figure 9: Comparison of viral RNA loads (Log_{10} RNA copies/mL) in respiratory samples of culture positive and negative samples.

The respiratory specimens are plotted by log_{10} RNA copies/mL (y-axis) and are stratified by SARS-CoV-2 cell culture results (positive, n = 15 negative, n = 48). Subgenomic RNA, Ag rapid FIA, and Ag Gold positive results are indicated as red data points (n = 25, 38, and 23, respectively), whereas negative (n = 41, 22, and 31 respectively) as white data points. In case of Ag Gold, the unavailability of samples is indicated as grey data points. The horizontal lines on x-axis indicate mean and 95% confidence interval.

C. Risk factors analysis for SARS-CoV-2 and association of vitamin D status with severity of COVID-19

i. Patient's demographic and clinical features

Between March 2020 and January 2021 a total of 136 participants were included for this analysis, including 95 patients with COVID-19 and 41 healthy individuals. The data of serum 25(OH) D was collected from all 136 participants, while serum concentration of $1,25(OH)_2$ D was obtained from 86 patients with COVID-19 and 32 healthy individuals (n = 118). The study cohort consisted of nearly equal male and female COVID-19 patients (49.5% vs 51.5%) with mean age of

64 ± 18.6 years, however, in comparison to COVID-19 patients, younger participants (40 ± 19.6 years, $p < 0.0001$) and female (24.4% vs 75.6%, $p = 0.0035$) are more pronounced in the healthy group. More than half of the patients (63.1%) presented at least one pre-existing comorbidity, with hypertension being the most frequent one followed by diabetes mellitus type II and dementia. Pulmonary comorbidities were infrequent; three patients had asthma (3.15%) and while only two (2.10%) had chronic obstructive pulmonary disease (COPD). Six patients (6.31%) were obese (BMI ≥ kg/m^2). Overall, 59% (n = 56) of patients presented with vitamin D deficiency and 21.05% (n = 20) with vitamin D insufficiency with 25 (OH) D serum level < 20 ng/mL and 20-30 ng/mL, respectively. Detailed baseline demographic and clinical features of this study cohort are described in Table 5.

ii. Characteristics of patients based on the severity of SARS-CoV-2 infection

According to the degree of infection, the patients were divided into four groups. Of 95 patients, nine were in asymptomatic, 54 were in mild to moderate, seven were in severe and 18 patients were in critical group. Both male and female participants were comparable in each group ($p = 0.451$). Younger patients were found in the asymptomatic group (38.7 ± 10.1 years); nevertheless, as the severity of the illness increased in the subsequent groups, the age of the individuals also increased significantly ($p < 0.001$), with patients > 80 years of age presented in the critical group. Serum ferritin levels were only available for a small percentage of patients (14.7%, n = 14), although the difference was significant ($p = 0.005$). The of CRP level also increased significantly in the progressive group ($p < 0.001$). The severe and critical group patients presented with largest proportion of co-morbidities ($p = 0.002$) in Table 6.

Table 5: Demographic and clinical characteristics of all patients with COVID-19 and healthy controls.

Variables	Patients (*N* = 95)	Controls (*N* = 41)	*p*-value
Sex (M/F, *n*, %)	47/48 (49.5/50.5)	10/31 (24.4/75.6)	0.0035
Age (years, mean, SD)	64 ± 18.6	40 ± 19.6	< 0.0001
BMI (mean, SD)	23.5 ± 3.8		
Ferritn µg/L (*n*, mean, SD)	14 (667.0 ± 627.3)		
CRP mg/dL (*n*, mean, SD)	83 (4.6 ± 6.1)		
D-dimer µg/L (*n*, mean, SD)	62 (641.1 ± 784.8)		
Patients with Comorbidity (*n*, %)	60 (63.1)		
Hypertension	33		
Angina pectoris	5		
Dementia	13		
Cerebrovascular accident	6		
Diabetes mellitus type II	16		
Asthma	3		
COPD	2		
Chronic renal failure	1		
Others	36		
25 (OH) D level ng/mL (*n*, mean, SD)	95 (20.3 ± 12.1)	41 (10 ± 5.2)	< 0.0001
1,25 $(OH)_2$ D pg/mL (*n*, mean, SD)	86 (73.7 ± 41.5)	32 (50.6 ± 22.4)	0.0036
Non Hyupovitaminosis [25 (OH) D > 20 ng/mL] (*n*, %)	39 (41)	1 (2.4)	< 0.0001
Hypovitaminosis [25 (OH) D ≤ 20 ng/mL] (*n*, %)	56 (59)	40 (97.6)	

BMI; body mass index (weight in kilograms divided by the square of the height in meters), CRP; C-reactive protein; COPD; chronic obstructive pulmonary disease SD; Standard deviations, 25 (OH) D; 25-Hydroxyvitamin D, 1,25 $(OH)_2$ D; 1,25-Dihydroxyvitamin D, *p*-values were calculated with Independent samples T-test.

Other comorbidities include; Arrhythmia, Arthritis, Aneurysm, Benign prostatic hyperplasia, Carcinoma of breast, lung, colon, prostate or thyroid, Dyslipidemia, Guillain-Barre syndrome, Glaucoma, Gout, Osteoporosis, Status epilepticus.

Table 6: Patient's characteristics based on the degree of SARS-CoV-2 infection

	Asymptomatic	Mild to Moderate	Severe	Critical	*p*-value
Patients (*n*)	9	60	8	18	
Sex (M/F, *n*, %)	5/4 (56/44)	27/33 (45/55)	6/2 (75/25)	9/9 (50/50)	0.451
Age (years, mean, SD)	38.7 ± 10.1	63.1 ± 18.5	64.4 ± 8.95	80.2 ± 8.36	< 0.001
BMI (*n*, mean, SD)	9 (23.6 ± 3.2)	58 (23.6 ± 4.18)	8 (24.3 ± 4)	18 (23.0 ± 4.24)	0.912
Ferritin (*n*, mean, SD)	–	7 (279.9 ± 154.2)	1 (2001 ± 0)	6 (896 ± 604)	0.005
CRP (*n*, mean, SD)	6 (0.81 ± 0.3)	51 (2.29 ± 3.08)	8 (6.19 ± 5.42)	18 (11.4 ± 7.88)	< 0.001
D-dimer (*n*, mean, SD)	6 (199.6 ± 159.2)	38 (461.8 ± 689.3)	4 (385.7 ± 115.6)	14 (970.3 ± 1132)	0.120
Patients with Comorbidity (*n*, %)	1 (11)	37 (62)	6 (75)	15 (84)	0.002
Hypertension	0	20	3	10	
Angina pectoris	0	5	0	0	
Dementia	0	7	0	6	
Cerebrovascular accident	0	4	1	1	
Diabetes mellitus type II	0	6	1	9	
Asthma	1	1	0	1	
COPD	0	1	0	1	
Chronic renal failure	0	0	1	0	
Others	0	22	5	9	

In-hospital mortality (*n*, %)	0	0	0	12	< 0.001
25 (OH) D level ng/mL (*n*, mean, SD)	9 (20.9 ± 8.7)	60 (18.9 ± 10.1)	8 (22.4 ± 17.9)	18 (23.7 ± 16.3)	0.478
1,25 $(OH)_2$ D pg/mL (*n*, mean, SD)	9 (83.9 ± 58.9)	54 (70 ± 53.9)	7 (96.6 ± 50.1)	16 (70.1 ± 44.7)	0.356

BMI; body mass index (weight in kilograms divided by the square of the height in meters), CRP; C-reactive protein; COPD; chronic obstructive pulmonary disease SD; Standard deviations, 25 (OH) D; 25-Hydroxyvitamin D, 1,25 $(OH)_2$ D; 1,25-Dihydroxyvitamin D. *p*-values were calculated with one-way analysis of variance (ANOVA)

Asymptomatic: Positive SARS-CoV-2 test, no symptoms; Mild to moderate: Mild symptoms no dyspnea or oxygen saturation ≥ 94% on room air at sea level; Sever: Oxygen saturation < 94% and requires high flow oxygen; Critical: respiratory failure, shock, requires mechanical ventilation or fatal.

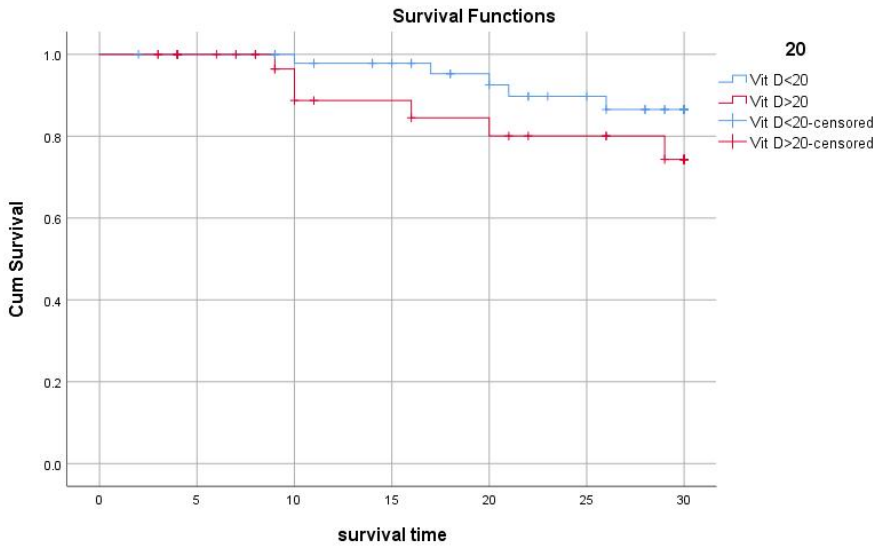
Other comorbidities include; Arrhythmia, Arthritis, Aneurysm, Benign prostatic hyperplasia, Carcinoma of breast, lung, colon, prostate or thyroid, Dyslipidemia, Guillain-Barre syndrome, Glaucoma, Gout, Osteoporosis, Status epilepticus.

iii. Association of serum 25 (OH) D and 1,25 $(OH)_2$ D levels with degree of severity

We adopted the cut-off concentration level of serum 25 (OH) D as < 20ng/mL for defining vitamin D deficiency. Our results indicated vitamin D deficiency in both the healthy group and the COVID-19 patient group. The mean concentration of 25 (OH) D (ng/mL) in healthy subjects was 10±5.2 and 20.3±12.1 in patients with COVID-19 ($p < 0.001$) (Table 5), whereas the mean concentration of 1,25 $(OH)_2$ D (pg/mL) was 50.6±22.4 and 73.7±41.5 in healthy subjects and COVID-19 patients ($p < 0.0036$), respectively (Table 5). The categorization of the patients on the basis of degree of severity also showed low levels of mean 25 (OH) D in all four group, however, the levels did not differ significantly among each group ($p = 0.478$) (Table 6). Similarly, serum concentrations of 1,25 $(OH)_2$ D did not differ significantly according to severity between the four groups ($p = 0.358$) (Table 6).

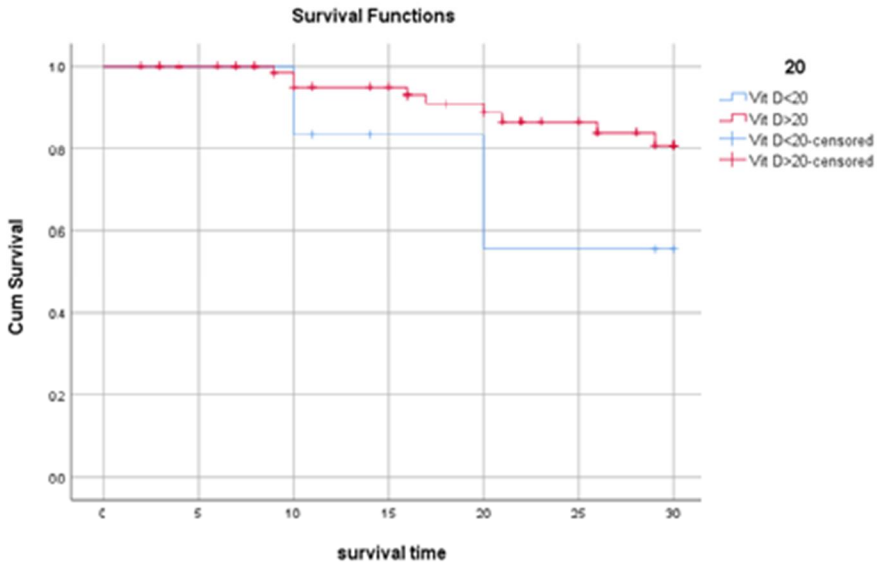
iv. Effect of serum 25 (OH) D and 1,25 $(OH)_2$ D levels with 30-day survival time

We also performed the analysis of 30-day survival time and divided the patients by serum concentration of 25 (OH) D (in ng/mL) and 1,25 $(OH)_2$ D (in pg/mL). The patients were analyzed according to < 20 vs ≥ 20 serum level of vitamin D. The mean 30-day survival time did not differ significantly at 28.6 (27.33-29.83) and 26.6 days (23.94-29.29) for the < 20 vs ≥ 20ng/mL concentration of 25 (OH) D, respectively (long-rank $p = 0.181$) (Figure 10). Likewise, the 30-day survival time was not significantly different when compared between < 20 vs ≥ 20pg/mL concentration of 1,25 $(OH)_2$ D [23.9 (17.22-30.56) vs 27.9 (26.44-29.29) days, log-rank $p = 0.164$, respectively] (Figure 11). Our results indicates that the severity of disease or the need of respiratory support was unrelated to the serum concentration of 25 (OH) D and 1,25 $(OH)_2$ D.



25 (OH) D < 20 ng/mL		25 (OH) D ≥ 20 ng/mL		*p*-value (Log Rank)
n	30-day Survival Time (Mean, 95% CI)	n	30-day Survival Time (Mean, 95% CI)	
56	28.6(27.33-29.83)	39	26.6(23.94-29.29)	0.181

Figure 10: Kaplan-Meier survival analysis according to the serum concentration of 25-hydroxyvitamin D in patients with COVID-19



1,25 $(OH)_2$ D < 20 pg/mL		1,25 $(OH)_2$ D ≥ 20 pg/mL		*p*-value (Log Rank)
n	30-day Survival Time (Mean, 95% CI)	n	30-day Survival Time (Mean, 95% CI)	
6	23.9 (17.22-30.56)	80	27.9 (26.44-29.29)	0.164

Figure 11: Kaplan-Meier survival analysis according to the serum concentration of 1,25-dihydroxyvitamin D in patients with COVID-19

VII. Discussion

Reliable and valid serological tests for SARS-CoV-2 are still insufficient, but they are urgently needed for effective diagnosis, contact tracing, epidemiologic elucidation, and vaccine development. Previous results from the SARS-CoV pandemic shown that assessing the antibody response was useful for serodiagnosis[66, 67]. We developed and tested an ELISA to detect anti-nucleocapsid antibodies (total Ab) utilizing rNP antigen produced in plants and *E. coli*, and compared its performance to that of three commercial ELISAs (SD Biosensor STANDARDTM E COVID-19 Total Ab ELISA and EDITM Novel Coronavirus COVID-19 IgG and IgM).

In both prokaryotic and eukaryotic hosts, nucleoproteins may be readily produced and purified in large quantities. Plant-based expression systems have several benefits over more extensively used insect or mammalian systems, such as optimized medium and low-cost growing conditions [68]. The benefits of plant-based expression systems over bacterial or yeast systems include post-translational changes that are comparable to mammalian cell lines and the absence of contaminating pathogens or endotoxins that can cause problems with protein purification. [68, 69]. The lack of precise protein glycosylation and the low yield of recombinant proteins are seen as drawbacks of employing plant-expressed proteins. However, *N. benthamiana* has recently become the preferred protein expression host due to its tolerance for higher levels of transient gene expression, rapid biomass generation, a defective post-transcriptional gene silencing system, and engineering strategies in the plant secretory pathway, all of which can overcome the difficulty of low yield [70, 71].

Using our ELISA with plant-expressed rNP, we demonstrated excellent sensitivity and specificity with high accuracy of total antibody testing for SARS-CoV-2 identification. Our results demonstrated the superior performance with plant-expressed recombinant nucleoproteins compared to all other ELISA assays. Interestingly, all patients seroconverted during the first two weeks, indicating 100% total Ab seropositivity by 10 days post symptom onset; these data supported those

of earlier published studies [72-74]. In a previous study, total Ab seroconversion was shown to be high (93.1%) when compared to IgG (82.7%) and IgM (64.7%) [74], which is consistent with our findings (92.91 %). According to a recent publication on the assessment of [75] , the sensitivity and specificity for detecting total Ab (90%, 100%) outperformed IgA (90%, 93%) and IgG (96%, 65%) alone. These findings are in line with our total Ab findings (92.91% and 94.30%). Despite using a more sensitive double sandwich ELISA technique [76] than our indirect ELISA method, the sensitivity of the antibody detection test found in the first week was 38.3 percent [74], which was quite low.

The results of our ELISA based on the detection of total Ab in COVID-19 patients suggested that it could be used in serosurveys to determine the mortality rate in various groups and to identify asymptomatic infections. It can also help in charting the kinetics of immune responses by tracking all antibodies generated during the course of illness.

The present study also demonstrates the performance characteristics of the Ag-RDTs for detecting SARS-CoV-2 in respiratory samples and describes the correlation between rRT-PCR and viable SARS-CoV-2. Our analytical findings revealed that both Ag-RDTs are comparable and were performed with a high specificity (100%) for detecting viable SARS-CoV-2 in respiratory samples; however, the overall sensitivity was lower (53.49%–74.51%) than that of rRT-PCR. Even though the correlation between transmissibility and viral load remains unclear, several studies have reported that samples with higher viral load of ≥ 6 $\log_{10}$ RNA copies/mL would be associated with infectivity in cell culture [23, 61, 77, 78]. Consistent with the findings of other studies, our results suggests that, Ag-RDTs although less sensitive, align efficiently with the cell culture-based techniques to identify infectiousness than rRT-PCRs [23, 79-81].

The key aspect of utilizing a point-of-care test is its ability to discriminate between noninfected and infected individuals who can potentially transmit the virus. Our correlation analysis revealed that both Ag-RDTs effectively identified all SARS-CoV-2 viable specimens (100%) that were positive in the cell culture. Notably, almost 26% (4/15) of the samples, despite having a relatively low viral load (< 6 $\log_{10}$ RNA copies/mL), still tested positive in cell culture

(Figure 9). Even though less sensitivity of Ag-RDTs was observed for low viral loads (< 6 $\log_{10}$ RNA copies/mL), these culture positive samples also tested positive with Ag-RDTs but not with sgRNA PCR assay. Although no direct evidence indicates that virus infectivity in the cell culture correlates with virus transmission in humans, a correlation was observed between the virus detection and communicable period in a golden Syrian animal model [82]; this is considered an indicator of infectivity. In the present study, Ag rapid FIA met the minimum performance requirement of WHO, which supports the use of SARS-CoV-2 Ag-RDTs with > 80% sensitivity and ≥ 97% specificity at a viral load of > 6 $\log_{10}$ RNA copies/mL (equivalent to Ct ≤ 28; N-gene) using the reference method of nucleic acid amplification [83].

Moreover, our analysis revealed that presence of viral sgRNA was not associated with infection in the cell culture. Furthermore, one study [61] reported that presence of sgRNA indicates active viral replication, and thus viral infection; however, two recent studies [78, 84] described that detection of sgRNA outlived the detection of infectious virus. This presumably occurred because sgRNA is associated with cellular membranes and is nuclease resistant, which makes it stable or protects it from the host cell response [84, 85]. Therefore, the presence of sgRNA is not a direct evidence of active infection; instead, the presence of sgRNA at a lower level than that of genomic RNA results in its detection for a relatively shorter period of time [84].

The present study also demonstrated the the level of disease severity and predictive fatal outcome with the status of vitamin D by measuring the two major metabolites; 25 (OH) D and 1,25$(OH)_2$ D in patients with COVID-19. It is crucial to highlight that age, gender, ethnicity, comorbidities, and co-infection are all possible risk factors for COVID-19 severity and fatality. A research in China looked at the influence of comorbidities on 1590 COVID-19 patients and found that COVID-19 patients with any comorbidity (hypertension, diabetes) had worse clinical results than those without [86].

The current findings reveal no significant association between serum levels of 25 (OH) D and 1,25 $(OH)_2$ D, diseases severity and the 30-day outcome of all patients with COVID-19. The serum levels of both vitamin D metabolites were unrelated to mortality and the need of ventilator

support. Despite the fact that both COVID-19 and healthy subjects of our study had lower levels of both vitamin D metabolites, we found no evidence that this contributes to their increased risk of disease severity or mortality.

The recent literature have consistently described that COVID-19 patients had lower 25(OH) D blood concentrations than non-infected controls and is related to the development of critical illness and high mortality rate [87, 88]. The potential benefit of vitamin D in the prevention of respiratory tract infection is more substantial [89], however, the clinical consequences of decreased vitamin D levels in COVID-19 patients are not well recognized. For instance, Cereda et al., observed no link between serum concentration of 25 (OH) D (tested within 48 hours of admission) and a variety of clinical characteristics. They discovered a positive relationship between 25(OH) D and in-hospital mortality after controlling for significant covariates, which contradicts the hypothesis that vitamin D insufficiency increases the likelihood of SARS-CoV-2 infection and a worse clinical outcome [90]. The authors further suggested that supplementation of vitamin D may potentially facilitate a severe course of COVID-19 by inducing an excessive macrophage response and a subsequent cytokine storm. Another study [91] investigated both vitamin D status marker and vitamin D degradation products; including serum concentrations of 25(OH) D_3, 25(OH) D_2, 24,25$(OH)_2$ D_3, and 25,26$(OH)_2$ D_3 and found that serum concentrations of all vitamin D metabolites and the vitamin D metabolite ratio (VMR) did not differ significantly between non-fatal and fatal group. Moreover, the requirement for ventilator support was also unrelated to levels of 25(OH) D vitamin D, the two vitamin D catabolites, and the VMR [91]. One study used UK Biobank data (2006-2010) for the status of vitamin D and ethnicity and correlated with COVID-19; the results found no potential link between levels of vitamin D and risk of COVID-19 [92]. Our findings contribute to the expanding body of research that shows no significant association between vitamin D concentrations and the severity or progression of COVID-19 [93-96]. For instance, in a retrospective case–control study of 216 COVID-19 patients and 197 controls, Hernández et al. did not find any relationship between vitamin D concentrations or deficiency and the severity of the disease. Another study analyzed 138 patients with COVID-19 and 82 healthy subjects. Despite the

fact that patients with SARS-CoV-2 infection had lower concentrations of 25 (OH) D than non-infected participants, however, they did not find higher incidence of SARS-CoV-2 infection in individuals with lower 25 (OH) D levels [97].

On the other hand, several studies showed significant relationship between the serum 25 (OH) D, rate of SARS-CoV-2 infection with severity and mortality of the disease [98-100]. At present, the inconsistent results of current studies do not corroborate vitamin D supplementation in patients with COVID-19. A recent study found that high dose of vitamin D supplementation reduces COVID-19 severity and mortality [101]. In contrast, a placebo-controlled intervention research using a single dosage of 200,000 IU of vitamin D_3 did not show a significant reduction in hospitalization time [102]. A recent editorial by Leaf and Ginde emphasized the lack of consistent evidence for the positive effects of vitamin D therapy in COVID-19 patients [103].

VIII. Conclusions

We determined that ELISA based on plant-expressed rNPs produced the most accurate findings. This study adds to our understanding that detecting total Ab against recombinant nucleoproteins of SARS-CoV-2 using ELISA has significant diagnostic utility as a serological test. The findings provide compelling support for the widespread use of plant-based total Ab serological tests in the complementary diagnosis and clinical monitoring of COVID-19 patients.

Our findings imply that Ag-RDTs can efficiently detect SARS-CoV-2-infected samples at the point-of-care level, particularly with moderate to high virus loads. Such point-of-care testing have the potential to improve public health policies for limiting virus spread. Despite their modest analytical sensitivity, the tests are inexpensive, and their widespread usage will be a crucial tool in suppressing community transmission, particularly in areas where molecular approaches are unavailable.

Patients with pre-existing illnesses such as cardiovascular disease, hypertension, chronic renal disease, and diabetes mellitus are predisposed to an adverse clinical course and a greater risk of intubation and mortality. Moreover, the current data do not show that vitamin D has a major role in the course, outcome, and mortality of COVID-19 in vitamin D deficient individuals.

Publications

Tariq, Misbah, Jun-Won Seo, Da Young Kim, Merlin Jayalal Lawrence Panchali, Na Ra Yun, You Mi Lee, Choon-Mee Kim, and Dong-Min Kim. "First report of the molecular detection of human pathogen Rickettsia raoultii in ticks from the Republic of Korea." Parasites & Vectors 14, no. 1 (2021): 1-5.

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